



## Intraspecific variability of nectar attracts different bats: the case of *Pseudalcantarea viridiflora*, a bromeliad with crepuscular anthesis

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Received: November 27, 2020

Accepted: April 2, 2021

### ABSTRACT

Intraspecific variation in floral traits, such as nectar, could lead to differences in the identity and pollinator effectiveness of visitors between populations of a flowering plant. We studied the bat-pollinated bromeliad species *Pseudalcantarea viridiflora* to determine whether there are differences in the identity of its pollinators and in its nectar traits between two study sites located at different latitudes in Veracruz, Mexico. We studied phenology and determined the volume, concentration and quantity of dissolved sugars in the nectar at both sites. The northern *P. viridiflora* population produced nectar that was more sugar-rich, leading to pollination by a larger species, *Anoura geoffroyi*, whereas the southern populations were visited mainly by *Glossophaga mutica*, a smaller pollinator. The most abundant nectarivorous bats at each site differ in terms of their efficiency as pollinators, and we suggest that this might affect their contribution to the fitness of the bromeliad.

**Keywords:** *Anoura*, Bromeliaceae, chiropterophily, *Glossophaga*, Mexico, nectar traits, pollination, pollinator effectiveness

## Introduction

The pollinator species of a given plant can change among sites (Boyd 2004; Lobo *et al.* 2012), as can the efficiency or effectiveness of pollinators (see King *et al.* 2013 for definitions related to this topic). All of these conditions may exert selective pressures on the phenotypic traits of the plant (Thompson 1994), including flowering phenology (Lobo *et al.* 2003), anthesis duration (Schlumpberger *et al.* 2009; Munguía-Rosas *et al.* 2010), floral morphology and size (Pérez-Barrales *et al.* 2007; Schlumpberger *et al.* 2009; Newman *et al.* 2014; Hattori *et al.* 2015), corolla coloration (Berardi *et al.* 2016), quantity of nectar (Schlumpberger *et al.* 2009; Brown *et al.* 2011; Gijbels *et al.* 2014) and the

plant reproductive system (Eckert 2002). Populations that receive different floral visitors could present differentially affected reproductive efficiency and pollen flow between flowering individuals (*i.e.*, Rovere *et al.* 2006). Moreover, in species with mainly nocturnal anthesis, some diurnal visitors may be capable of pollinating the plant at one site but not at another (Fleming *et al.* 1996; Schlumpberger *et al.* 2009; Aguilar-Rodríguez *et al.* 2016), highlighting the need to evaluate the role of diurnal *vs.* nocturnal floral visitors as pollinators.

Zoophilous flowering plants are typically pollinated by a subset of pollinators from the same functional group that share flower visitation behavior, morphological traits and pollination probability (*i.e.*, perching birds, hovering

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birds, long-tongued flies or bees, nectarivorous hovering bats, etc.), rather than by a particular species (Fenster *et al.* 2004; Ashworth *et al.* 2015). However, members of the same functional group can differ in terms of their efficiency as pollinators between plant species (Javorek *et al.* 2002; Tschapka 2003; Brown *et al.* 2011; Watts *et al.* 2011; Rocca & Sazima 2013; Rogers *et al.* 2013; Stewart & Dudash 2017b), probably due to ethological differences (floral constancy, number of visits performed, search time and handling time at the flowers, temporal variation of visits, pollen transfer efficiency, grooming behavior; Fishbein & Venable 1996; Dafni *et al.* 2005; Fumero-Cabán & Meléndez-Ackerman 2007; Hoehn *et al.* 2008; Willmer 2011), or as a result of the morphological fit between the body of the pollinator and the arrangement of floral parts (*i.e.* Solís-Montero & Vallejo-Marín 2017). These “pollinator landscapes” impose selective pressures that may dictate floral ecotypes (Newman *et al.* 2014), in which floral traits and pollinator characteristics would match locally, therefore influencing pollination outcome (Willcox *et al.* 2017). For example, the presence or absence of the putative pollinator –in addition to other environmental factors– may correspond to geographic variation in the reproduction of plant species (Fleming *et al.* 1998; Quesada *et al.* 2004; Munguía-Rosas *et al.* 2010) via processes such as differential pollen limitation among populations through pollinator activity/abundance (Cosacov *et al.* 2008; Fernández *et al.* 2012).

The family Bromeliaceae comprises about 3700 species of 79 genera (Butcher & Gouda *et al.* 2021), with half of the species epiphytic in habit (Zotz 2013). In this family, pollination mainly occurs through visits by the hummingbird (Kessler & Krömer 2000; Krömer *et al.* 2006; Kessler *et al.* 2020). However, the plants of some genera, especially those of the subfamily Tillandsioideae (*e.g.*, *Guzmania*, *Tillandsia* and *Vriesea*), have evolved diverse floral characteristics that attract different pollinator groups such as insects, birds and bats (Benzing 2000; Kessler & Krömer 2000; Givnish *et al.* 2014; Aguilar-Rodríguez *et al.* 2016).

Within the bromeliads, bat pollination has been recorded in more than 40 species of 10 genera (Aguilar-Rodríguez *et al.* 2019a). The characteristics shared with other bat-pollinated plants include crepuscular/nocturnal anthesis, pale coloration of the petals, abundant, diluted (often <20%) and hexose-rich nectar, with a floral scent containing sulfurous compounds (Bestmann *et al.* 1997; Baker *et al.* 1998; Tschapka & von Helversen 2007; Krömer *et al.* 2008). Information about the existence of geographic variation in the pollinator fauna of bromeliads is scarce. However, Sazima *et al.* (1999) described *Vriesea gigantea* Gaudich. as chiropterophilous, while Paggi *et al.* (2015) described the same species, at another Atlantic forest site in Brazil, as having diurnal anthesis and observed pollination by hummingbirds and bees.

Nectar production can consume up to 37 % of the daily product of photosynthesis (Southwick 1984) and

nectar production patterns are therefore considered to be under strong selective pressure (Heil 2011; Willmer 2011). In particular, members of Bromeliaceae expend large amounts of organic and inorganic compounds in their nectar (Göttlinger *et al.* 2019) and some species have developed adaptations in order to reduce nectar secretion following pollination (*e.g.*, once pollen is removed and/or deposited; Ordano & Ornelas 2004). Bats are energetically expensive pollinators for the plants, due to their large size (compared to other pollinators) and high metabolism (von Helversen & Reyer 1984). Nectarivorous bats must obtain at least 5  $\mu$ l of nectar of 20 % sugar concentration from each flower just to recover the metabolic energy invested in the visit (Nassar *et al.* 1997). Since nectar is the main reward offered by the bromeliads to the pollinators (Benzing 2000), we hypothesize that it could be the floral trait most likely to reflect any differences in the energetic preference of the pollinators in a given population.

The genus *Pseudalcantarea* comprises three species: *P. grandis*, *P. macropetala* and *P. viridiflora* (Barfuss *et al.* 2016). These species have helicoiform corollas formed by strap-shaped, twisted petals of pale colors, with stamens and style extending beyond the petals (Beaman & Judd 1996; Krömer *et al.* 2012). They produce hexose-rich nectar, suggesting pollination by bats (Krömer *et al.* 2008). In this genus, pollination by bats has been reported previously in *P. macropetala* (Aguilar-Rodríguez *et al.* 2014) and in *P. viridiflora* (Aguilar-Rodríguez *et al.* 2019b), while preliminary information pertaining to *P. grandis* suggests that it is also a chiropterophilous species (P. A. Aguilar-Rodríguez unpublished data).

In this paper, we examined pollination in the epiphytic species *P. viridiflora* in order to determine whether there are differences in the identity or importance of its main pollinators (bats) between two study sites with tropical montane cloud forest (TMCF) vegetation in the central and southeastern region of Veracruz state, in Mexico. These two sites present different richness in the nectarivorous bat species that could serve as potential pollinators of this bromeliad (Sosa *et al.* 2008; Coates *et al.* 2017). We hypothesized that the bats serve as the only pollinators of *P. viridiflora* at both sites, but that a greater number of bat species will fulfill this role at the southeastern locality with the highest species richness.

## Materials and methods

### Study area

The first study site is located in the central region of Veracruz state, at Rancho Viejo (19°31'28.99" N, 96°59'3.99" W, 1480 m a.s.l.), in the Tlalnelhuayocan municipality (Fig. S1 in supplementary material). The climate is temperate-humid, with an annual mean



temperature of 14 °C and an annual precipitation of 1650 mm, with a drier period between November and April (Muñiz-Castro *et al.* 2006). The natural vegetation is TMCF, which is composed of trees such as *Quercus* L. spp. (Fagaceae), *Liquidambar styraciflua* L. (Hamamelidaceae) and *Clethra mexicana* DC. (Clethraceae), as well as shrubs, hemiepiphytes and epiphytes (Zamora & Castillo-Campos 1997; Toledo-Aceves *et al.* 2014). The site is a fragment of secondary forest on private property close to a river, where individuals of *P. viridiflora* are found together with other bromeliads, such as *T. multicaulis* Steud. and *P. macropetala*. In the study area, 26 species of bats are reported, three of them nectarivorous (Sosa *et al.* 2008).

We compared the data obtained in the first site (Rancho Viejo) with the information collected previously in a second locality (hereafter, Los Tuxtlas; Aguilar-Rodríguez *et al.* 2019b). This second study site is located in the southeastern region of the state, in the ejido of Adolfo Ruiz Cortines (18°32'3.55"N, 95°8'18.54"W, 1030 m a.s.l.; Fig. S1 in supplementary material), in the municipality of San Andrés Tuxtla, on the southeastern slope of the volcano San Martín Tuxtla, which forms part of the Los Tuxtlas Biosphere Reserve. Elevation of the study area ranges between 1050-1100 m a.s.l. The climate is temperate and humid, with an annual mean temperature of 18 °C and 40 mm of average precipitation per month, or 4000 mm per year (Soto 2004), and a drier season between March and May. The vegetation is composed of remnants of TMCF, derived secondary forests and pastures (Castillo-Campos & Laborde 2004). The most common tree genera are *Carpinus*, *Ulmus*, and *Quercus*, whereas the understorey is characterized by *Chamaedorea* palms, (tree) ferns and epiphytes (Krömer *et al.* 2013). The floral observations were conducted in a fragment of secondary forest (18°32'3.55"N, 95°8'18.54"W, 1030 m a.s.l.; Fig. S1 in supplementary material) in which *Saurauia scabrida* (Actinidiaceae) predominates. This fragment connects with a larger TMCF remnant, in which individuals of *P. viridiflora* occur mostly solitary at heights of between 1.50 and 5 m. In the Los Tuxtlas region, at least 65 bat species are reported, six of them as nectarivorous (Coates *et al.* 2017).

We selected these sites because both have similar altitudes, remnant trees of TMCF, and had enough individuals of *P. viridiflora* (over 100 individuals per hectare at both sites) to perform the pollination experiments and record pollinator's visits (over 100 individuals per hectare at both sites). Additionally, they offered security and accessibility to carry out the experiments *in situ*.

### Study species

*Pseudalcantarea viridiflora* is an epiphytic bromeliad that forms a tank of almost 40 cm in diameter. Its green leaves are 25-50 cm in length. Its inflorescence is simple and 45-50 cm in length and *ca.* 1 cm in width. The flowers are

actinomorphic, with a helicoiform corolla. The subsessile flower petals are light green or cream, strap-shaped and twisted, of 9 cm in length and 1 cm in width (Fig. 1). Stamen length exceeds that of the petals. The plant develops a fusiform fruit capsule of *ca.* 6 cm in length and 1 cm in width that contains seeds adapted to wind dispersal (Krömer *et al.* 2012). *Pseudalcantarea viridiflora* is distributed in Mexico, Guatemala, Honduras and Nicaragua (Espejo-Serna *et al.* 2005). This species, at least in the south of Veracruz state, is pollinated by nectarivorous bats (Aguilar-Rodríguez *et al.* 2019b). Plants used in this study were collected with the permission of the Secretaría del Medio Ambiente y Recursos Naturales (permit No. 02405/14 granted to MCMG). Voucher specimens of *P. viridiflora* collected at both study sites, Rancho Viejo (PAAR 10) and Los Tuxtlas (PAAR 9), were deposited at the herbarium XAL of the Instituto de Ecología, A.C. in Xalapa, Veracruz.



**Figure 1.** **A)** Flowering individual of *Pseudalcantarea viridiflora* in the TMCF of the San Martín Tuxtla volcano. **B)** Flower of *P. viridiflora*. The arrow indicates the stigma. White bar = 1 cm. Photographs: **A**, Thorsten Krömer; **B**, Pedro A. Aguilar-Rodríguez.

### Anthesis and flowering

We conducted the fieldwork during the peak of the *P. viridiflora* flowering period, in October 2015 at Rancho Viejo, and between the end of August and beginning of September 2014 at Los Tuxtlas (for the latter, see Aguilar-Rodríguez *et al.* 2019b). At both sites, we translocated specimens to accessible tree trunks and individually marked them to facilitate a continuous monitoring of the floral buds until fructification (Ollerton & Dafni 2005). Beginning of anthesis was considered as the moment at which the petals opened to reveal the reproductive organs of the flower, while senescence was considered to have occurred when the floral parts lost turgidity and stigmatic receptivity ceased (Aguilar-Rodríguez *et al.* 2014). We estimated the period of stigmatic receptivity by submerging stigmas in hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) every two hours during the whole anthesis (Martén-Rodríguez & Fenster 2008).



### *Volume and concentration of nectar*

We took all the nectar measurements *ex situ*, by taking at least 10 specimens of each population to a roofed nursery in the city of Xalapa, Veracruz (located about 11 km from the Rancho Viejo site at similar elevation), to measure nectar production data. After translocation of specimens to this nursery, they were allowed to adapt to the local conditions for at least one week, during which the plants were maintained in a natural light: darkness regime and supplied with abundant water. Only after this habituation period, we started with the samples collection.

We determined the patterns of production and concentration of nectar. In order to calculate nectar volume, we used glass capillary tubes of 10 and 80  $\mu\text{l}$  to extract accumulated nectar from one flower every two hours in the same flower (standing crop collection), beginning two hours after initiation of anthesis, and continuing until the measured volume reached zero (Aguilar-Rodríguez *et al.* 2014; 2016). The sugar concentration was measured in Brix degrees using a handheld refractometer (Mod. HRT32, range: 0-32 %, weight/weight, precision: 0.2 %; A. Krüss Optronic, Germany), every two hours. We obtained the total volume of nectar produced per flower by summing the values of production measured across all of the two-hour periods (Tschapka & von Helversen 2007). Later, we calculated the milligrams (mg) of dissolved sugars in the nectar using conversion tables and techniques explained by Kearns & Inouye (1993) and the work of Galetto & Bernardello (2005).

In bat-pollinated plant species, the nectar values at the end of the night are always extremely low in volume and concentration (*e.g.*, Tschapka & von Helversen 2007; von Helversen & Winter 2003; Martín-Rodríguez & Fenster 2008; Munguía-Rosas *et al.* 2010; Aguilar-Rodríguez *et al.* 2014; 2016), and it is doubtful whether the bats are still able to extract those low amounts of nectar volume. To get a more typical value (*i.e.*, one that is on average experienced by the bats during their visits) and not over-represent the low values typically obtained later in the night, we only took into account the values from the 80 % of the cumulative nectar production of each flower (the last 20 % of extremely low production, were omitted) to obtain the mean volume, concentration, and mg of sugars of both populations. Later, we compared them conducting a rank-sum test (t-test) using the program SigmaPlot ver. 12 (Badashah & Nath 2007).

### *Breeding system*

Breeding and mating could vary among plant populations (Müller *et al.* 2000; Rovere *et al.* 2006; Whitehead *et al.* 2018). We determined the breeding system of *P. viridiflora* in a previous study using three manual pollination treatments (Aguilar-Rodríguez *et al.* 2019b). However, we repeated these treatments in the Rancho Viejo population to account for any variation. All individuals used for this purpose were excluded from visits by potential pollinators. The

following treatments were conducted on  $\geq 10$  flowers each, from at least 10 individuals, respectively: 1) Spontaneous self-pollination (a non-manipulated flower); 2) Xenogamy (cross-pollination with pollen from another individual); 3) Experimental autogamy (controlled self-pollination with pollen of the same flower). For each treatment, we calculated the fruit set (percentage of fructification based on the number of flowers that had developed fruits), assessed four to eight weeks after the experiments. Later, we compared the seed set per fruit following the different treatments. To assess any differences between fruit set and seed set within both populations, as well as any interaction with the study site, we used a GLM with a binomial distribution and a logit function using the program R (R Development Core Team 2012). The pollination treatment was treated as a fixed effect, while fruit production (presence or absence) as a binary response variable, or seed number as a continuous response variable.

To establish whether *P. viridiflora* is self-compatible at Rancho Viejo, we obtained the Index of Self-Incompatibility (ISI) using the formula presented in Lloyd (1965), by dividing the ratio of seed set from the Autogamy treatment and the Xenogamy treatment (Zapata & Arroyo 1978), where values of between 0.30 and 1.00 indicate self-compatibility (Kamke *et al.* 2011). This index was also obtained using the percentage of fructification (fruit-set; Raduski *et al.* 2012) as an independent variable in these treatments since using both measures better reflects the reproductive success of the plant at two levels of development (Martinelli 1994; Wendt *et al.* 2002). To determine if the bromeliads from both sites suffered from limited pollen supply, the Pollen Limitation Index (PLI) was calculated per site, as shown in Larson & Barret (2000) and Becker *et al.* (2011). We used zero as the lower bound for the index and employed the fruit set values for this calculation. In this way, zero value means no limitation, and the closer the number approaches one, greater is the limitation.

### *Capture of bats and collection of pollen*

We carried out mist net sampling to identify the bat species that may potentially pollinate *P. viridiflora*. Two mist nets (height 3 m; length 6 m and 12 m) were placed near flowering bromeliads (Martinelli 1994; Kaehler *et al.* 2005; Tschapka & von Helversen 2007) or at sites serving as flight paths for bats, such as corridors, roads or openings in the vegetation (Wilson *et al.* 1996). Netting ceased after two hours of no captures (Santos-Moreno *et al.* 2010) and we avoided netting around full moon, since the activity period of the bats is shorter ("lunar phobia"; Morrison 1978). The nets were moved at least every second night of sampling to minimize learned mist-net avoidance (Marques *et al.* 2013). Sampling effort was measured as suggested by Straube & Biaconi (2002). At minimum, we sampled for three hours per night at each site, starting just after dusk. For logistic and climatic reasons, the sampling effort differed in the two



sites, at Rancho Viejo, the effort was 1014 m<sup>2</sup>/h/net, while in Los Tuxtlas this value was 19469 m<sup>2</sup>/h/net.

Using the field guides of Medellín *et al.* (2008) and Reid (2009), we identified the captured bats, following the classifications of Simmons (2005), Cirranello *et al.* (2016) and Calahorra-Oliart *et al.* (2021). We examined the captured individuals for the presence of pollen on the fur (Caballero-Martínez *et al.* 2009) to corroborate that these bats were visiting and transporting the pollen of *P. viridiflora*. To do this, we collected pollen with a paintbrush moistened with alcohol (Aguilar-Rodríguez *et al.* 2014; 2016). The pollen grains were kept with the bristles of the brush in plastic vials with at least 1.5 mL of 70 % ethanol (Voigt *et al.* 2009). Pollen samples were taken from the anthers of three individuals of *P. viridiflora* and kept in plastic vials with 70 % ethanol for comparison (size, shape, ornamentation) with the samples taken from the bat fur (Herrera & Martínez-del-Río 1998; Muchhala & Jarrín-V. 2002) using a microscope at 100x and 400x (Zeiss, Germany, Model: 426126). Determining where and approximately in what quantity are pollen grains of *P. viridiflora* transported by each bat species (which differ in body size), would help us to analyze the results of pollen occurrence (see below) and determine the efficiency of these species as pollinators.

We determined the occurrence of pollen-based on a criterion of presence/absence (Voigt *et al.* 2009). Each plastic vial was agitated to suspend the pollen grains and six drops were extracted and analyzed under the microscope. In each drop, we quantified the grains present using the “scanning” technique (*sensu* Caballero-Martínez *et al.* 2009), to avoid repeated quantification of the same element (Wendt *et al.* 2002). Then, we calculated the frequency of occurrence (Muchhala & Jarrín-V. 2002) of the bromeliad pollen morphotype (Silva *et al.* 2016; Souza *et al.* 2017), qualifying each sample as positive on the encounter of at least three pollen grains (Heithaus *et al.* 1975) in each microscope field. Finally, the positive samples were summed, and we calculated the percentage relative to the total pollen grains from that bat species using all individuals captured (Caballero-Martínez *et al.* 2009).

### Observations and efficiency of floral visitors

We recorded the nocturnal visitors, while the diurnal visitors were directly observed (Tschapka & von Helversen 2007; Schmid *et al.* 2011b), in October 2015 at Rancho Viejo and between August and September 2014 at Los Tuxtlas (see Aguilar-Rodríguez *et al.* 2019b). The nocturnal recordings were made with a camera with night-vision capability (DCR-SR65 Sony Corporation, Japan) and an infrared light (HVL-HILR Sony Corporation, Japan), which was installed on a tripod at a 1 m distance from the subject flower. Recordings started at dusk and lasted 3-3.5 hours, which covers the peak of activity of phyllostomid bats (Quesada *et al.* 2003). We inferred the identity of the species of bats by considering their relative size, visible

morphological traits, and behavior demonstrated during the visit and the presumably occurring bat species in the study zone, based on the literature (Medellín *et al.* 2008). Diurnal observations were made in the morning and in the late afternoon and lasted for two hours on each occasion. Observations started in the morning, 30 minutes after dawn, and lasted in the evening until just before dusk. A single observer monitored the plants at a distance of 2-3 meters.

A total of 47 and 32 hours of nocturnal recordings were made in flowers of *P. viridiflora* at the Rancho Viejo and Los Tuxtlas sites, respectively ( $n=15$  flowers, 7 individuals at Rancho Viejo;  $n = 13$  flowers, 7 individuals at Los Tuxtlas). At Rancho Viejo, recording lasted from 19:00 h to 01:30 h. At Los Tuxtlas recording began at 19:00 h and finished at between 22:00 h and 22:30 h due to climatic conditions (*i.e.*, frequent rains later at night). A total of 12 hours of diurnal observation was carried out at Rancho Viejo, and eight hours at Los Tuxtlas ( $n = 13$  flowers of 9 individuals, and 16 flowers of 7 individuals, respectively). This was conducted between 07:00 h and 11:00 h, and between 17:30 h and 18:40 h, respectively.

Due to the helicoiform shape of the corolla, all animals that made contact with the base of the petals and the aperture of the calyx were classified as floral visitors, but only the visitors that made contact with the reproductive organs of the flower were considered pollinators, regarding their consumption of nectar, pollen or neither. The data recorded included a) the frequency of visits: percentage of visits, in relation to the total number of visits made by this visitor (Schmid *et al.* 2011a), b) the frequency of visits per flower and per hour (Fleming *et al.* 1996), c) the number of visits in which the visitors possibly pollinated the flower and d) the behavior of each visitor. The duration of the visit was defined as the length of time for which the mouth-parts of the pollinator remained within the corolla (Muchhala 2006).

The efficiency of the floral visitors was determined through the exclusion of different floral visitors in the field (Wendt *et al.* 2001; Aguilar-Rodríguez *et al.* 2014; 2016; 2019b), conducted on 10-20 flowers per treatment. We used mainly the concept of the efficiency presented in Montalvo and Ackerman (1986) and Dafni *et al.* (1987), the percentage of fruit and seed set attributed to a specific activity period of different pollinators, as well as the observation of visitor behavior during the visit to the flowers. As preliminary observations (Aguilar-Rodríguez *et al.* 2019b) showed that diurnal visitors did not make any contact with the reproductive parts of the flowers and that these visitors were scarce, the following treatments were conducted in both populations: 1) Nocturnal Exposure (NE), in which the stigma of the flower was exposed during the night, but covered with a plastic tube (*ca.* 3 x 0.5 cm) held with cotton during the day, held with cotton so that the flower could only be pollinated by nocturnal visitors; 2) Emasculated



Nocturnal Exposure (ENE), which was conducted as with the previous treatment, but with the removal of the anthers (previously to anthesis to prevent self-pollination); this treatment prevents self-pollination and sires seeds only by nocturnal floral visitors (most likely bats). Finally, in a 3) Control treatment, flowers were constantly exposed to pollinators. From each treatment, we obtained the fruit set, which was compared in a similar manner to the results of the treatments for determining the breeding system.

To compare the relative pollination efficiency of the different visitors of *P. viridiflora*, the procedure described by Fleming *et al.* (1996) was adopted. In this calculation, the fruit set of the treatment ENE was divided by the fruit set of the control treatment, which in turn, was divided by the rate of visits/flower/hour of the pollinator of each population. In this estimate, a pollinator will score a higher effectiveness value when a lower number of visits is sufficient to reach a similar percentage of fructification as that obtained in the control treatment (Fleming *et al.* 1996). We used the ENE treatment in this calculation because *P. viridiflora* opens its flower with pollen unless we emasculate it.

## Results

### Anthesis and flowering

At Rancho Viejo, *P. viridiflora* flowered from the end of September until the end of November. At this site, each individual had 13 flowers (mean  $\pm$  SD:  $13.36 \pm 4.90$ ; range: 6-25;  $n = 11$  individuals), with  $1.09 \pm 0.30$  flowers opening every 1.5 days. At Los Tuxtlas, *P. viridiflora* flowered from July to October, although some individuals were still observed flowering in December. On average, individuals of *P. viridiflora* had 15 flowers each at this site ( $15.4 \pm 3.44$ ; range: 10-22;  $n = 10$  individuals), with  $1.06 \pm 0.25$  flowers opening every 2.08 days.

Elongation of the floral bud began *ca.* 36 hours before anthesis. Anthesis initiated with the opening of the petals and release of the stamens and style at 18:00 h at Rancho Viejo ( $n = 17$  flowers) and 19:00 h ( $n = 37$  flower) at Los Tuxtlas. Dusk occurred at both sites between 19:00-19:15 h. Dehiscence of the anthers coincided with the beginning of anthesis, while receptivity of the stigma began between 20 and 30 minutes afterward in both populations. The stigma produced a secretion that made it look "moist" and dark green while it was receptive.

The anthers were generally free of pollen and curved by 08:00-09:00 h on the following morning (up to 15 hours after anthesis). Receptivity of the stigma ceased between 10:30-11:00 h, 15-17 hours after becoming receptive ( $n = 10$  flowers, 6 individuals from both sites). The anthers began to open even before the petals had completely separated, which caused grains of pollen to adhere to the base of the

not yet receptive stigma, before the complete opening of the flower.

### Nectar volume and concentration

The Rancho Viejo site produced  $179.68 \pm 156.75 \mu\text{l}$  of nectar (CV: 87.24 %), with an average concentration of dissolved sugars of  $10.73 \pm 2.54 \%$ , or an average of  $29.68 \pm 32.38$  mg of sugar per flower ( $n = 9$  flowers from 5 individuals). At the Los Tuxtlas site, the volume of nectar was  $101.56 \pm 74.66 \mu\text{l}$  (CV: 73.51 %), with an average concentration of  $8.88 \pm 3.92 \%$  or an average of  $12.20 \pm 11.69$  mg of sugar per flower ( $n = 11$  flowers from 4 individuals). The average nectar volumes, and mg of sugars contained in the nectar were significantly different between sites (volume:  $t = 2.33$ ,  $df = 12.02$ ,  $p = 0.0037$ ; mg of sugar:  $t = 2.60$ ,  $df = 10.51$ ,  $p = 0.0254$ ), while concentration was not significantly different (concentration:  $t = 1.16$ ,  $df = 17.91$ ,  $p = 0.258$ ).

At both sites, nectar production began probably shortly after 18:00 h, but was not noticeable or measurable before 20:00 h. At this time, it reached its maximum volume and concentration ( $341.93 \pm 164.94 \mu\text{l}$  and  $12.83 \pm 2.06 \%$  at Rancho Viejo vs.  $143.86 \pm 62.66 \mu\text{l}$  and  $12.05 \pm 3.43 \%$  at Los Tuxtlas) and then diminished progressively throughout the night, lasting until approximately 04:00 h (Fig. 2A-B). The mg of dissolved sugars in the nectar (maximum:  $55.47 \pm 23.64$  mg and  $19.00 \pm 10.91$  mg at Rancho Viejo and Los Tuxtlas, respectively) also decreased overnight.

### Breeding system and experiments of exclusion of visitors

*Pseudalcantarea viridiflora* was found to be self-compatible at both sites (ISI=1.01 at Rancho Viejo, and ISI=0.97 at Los Tuxtlas). Only in the experimental Autogamy and Control treatments of the Rancho Viejo population did the fruit set reach 100%, and no treatment at either site was below 50% (Tabs. 1, 2). The fruit set values for the Control treatments between populations were different ( $U = 294.5$ ,  $Z = 729.50$ ,  $p = <0.001$ ), with that produced by natural pollination at the Rancho Viejo population higher than that found at Los Tuxtlas.

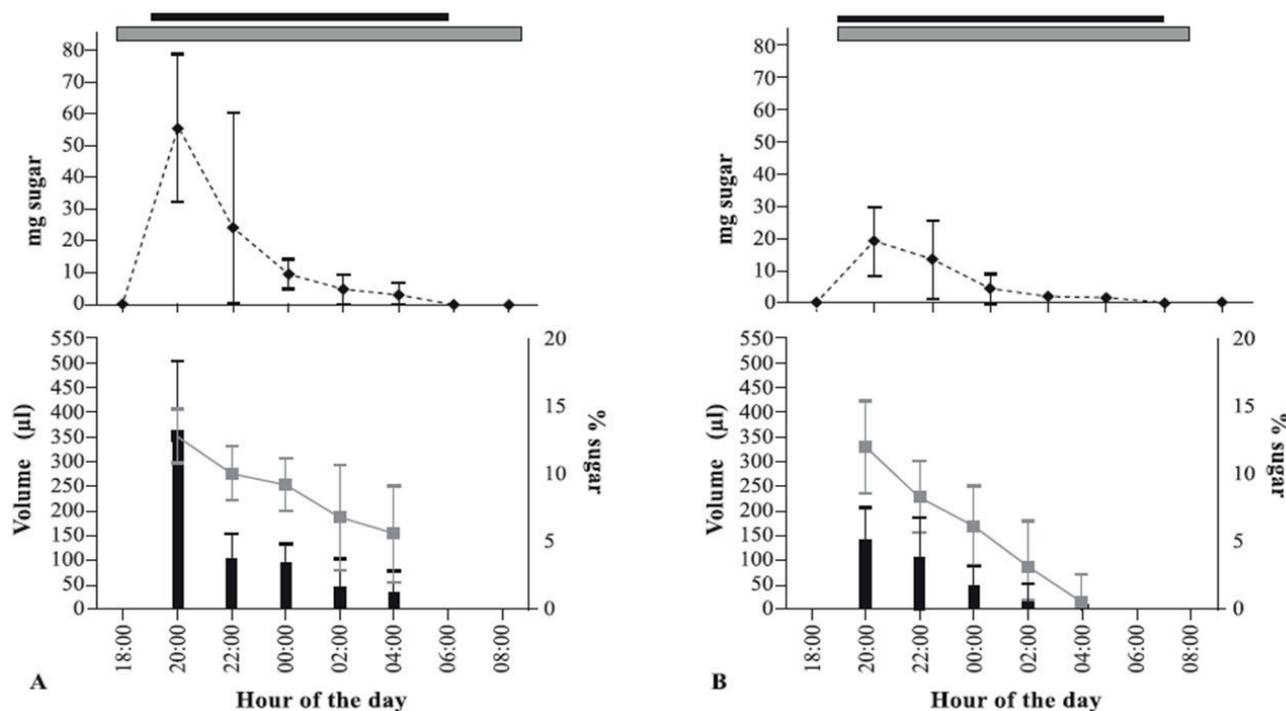
Differences in the fruit set and seed set within sites were found at Los Tuxtlas, where fruit set from Spontaneous Self-pollination and the ENE treatments were significantly different compared with the other treatments in the same population (GLM: Treatment Spontaneous Self-pollination: Estimate = -0.89, SE = 0.41,  $df = 5$ ,  $p = 0.0096$ ; ENE: Estimate = -0.98, SE = 0.40,  $df = 5$ ,  $p = 0.0048$ ). The GLM showed a significant effect between treatment and study site also for the Autonomous Self-pollination treatment and the NE treatment (GLM: Spontaneous Self-pollination\*Site: Estimate = 0.64, SE = 0.41,  $df = 5$ ,  $p = 0.0342$ ; NE\*Site: Estimate = 0.76, SE = 0.439,  $df = 5$ ,  $p = 0.0214$ ). In addition, seed set was statistically significant in the



## Intraspecific variability of nectar attracts different bats: the case of *Pseudalcantarea viridiflora*, a bromeliad with crepuscular anthesis

Experimental Autogamy, ENE and NE treatments at Los Tuxtlas (GLM: Experimental Autogamy: Estimate = 391.91, SE = 86.73, df = 5,  $p < 0.0001$ ; NE: Estimate = -343.45, SE = 83.98, df = 5,  $p < 0.0001$ ; ENE: Estimate = -369.21, SE = 87.67, df = 5,  $p < 0.0001$ ), while we also found a significant effect between treatment and study site in the seed set of the treatments Spontaneous Self-

pollination and the ENE treatments at Los Tuxtlas (GLM: Spontaneous-Self-pollination\*Site: Estimate = 266.11, SE = 88.70, df = 5,  $p = 0.0031$ ; Control\*Site: Estimate = -267.27, SE = 62.31, df = 5,  $p < 0.0001$ ). According to the values obtained for the Pollen Limitation Index, there is no pollen limitation in either of the study sites (PLI: Rancho Viejo = 0; Los Tuxtlas = 0.12).



**Figure 2.** Production of nectar in *Pseudalcantarea viridiflora* at two study sites. *Above:* Quantity of dissolved sugar in the nectar of each population throughout anthesis. Horizontal bars in the upper part indicate the longevity of the flower (dark grey) and the nocturnal period at the study site (black line). *Below:* Volume (black bars) and concentration (grey line) of sugars in the nectar throughout anthesis at two study sites: **A)** Rancho Viejo, **B)** Los Tuxtlas. Vertical lines indicate the SD.

**Table 1.** Results of the pollination treatments conducted with *P. viridiflora* flowers to determine the breeding system.

Treatment/Site	Flowers manipulated		% Fruit set (N)		Seed set (means±SD)	
	Rancho Viejo	Los Tuxtlas	Rancho Viejo	Los Tuxtlas	Rancho Viejo	Los Tuxtlas
<b>Spontaneous self-pollination</b>	10	14	50.00 (5)	64.29 (9) *	778.40 ± 428.07 <sup>a</sup>	1165.67 ± 358.47 <sup>A</sup>
<b>Xenogamy</b>	15	16	73.33 (11)	75.00 (12)	1209.18 ± 445.77 <sup>a</sup>	967.58 ± 354.10 <sup>A</sup>
<b>Experimental Autogamy</b>	11	14	100.00 (11)	78.57 (11)	1219.27 ± 512.94 <sup>a</sup>	930.36 ± 485.96 <sup>B</sup>
<b>Control</b>	31	29	100.00 (31)	65.51 (19)	1048.22 ± 468.52 <sup>a</sup>	521.42 ± 326.70 <sup>A</sup>

Different letters within the columns of average numbers of seeds per population (lower case for Rancho Viejo, upper case for Los Tuxtlas) indicate significant differences ( $p < 0.05$ ) in the seed set or fruit set (\*) within that population. We used 10 individuals for the Los Tuxtlas population, and 14 individuals for Rancho Viejo population.

**Table 2.** Results of the *in situ* pollination treatments conducted with *P. viridiflora* flowers to determine pollinator effectiveness.

Treatment/Site	Flowers manipulated		% Fruit set (N)		Seed set (means±SD)	
	Rancho Viejo	Los Tuxtlas	Rancho Viejo	Los Tuxtlas	Rancho Viejo	Los Tuxtlas
<b>NE</b>	15	12	66.66 (10)	83.33 (10)	447.4 ± 456.585 <sup>a</sup>	217.5 ± 264.64 <sup>A</sup>
<b>ENE</b>	15	10	60.00 (9)	50.00 (5) *	289.67 ± 344.66 <sup>a</sup>	508.40 ± 366.57 <sup>B</sup>
<b>Control</b>	31	29	100.00 (31)	65.51 (19)	1048.22 ± 468.52 <sup>b</sup>	521.42 ± 326.70 <sup>C</sup>

Abbreviated treatment names: NE: Nocturnal exposure, ENE: Emasculated Nocturnal Exposure. Number of seeds is shown as mean ± standard deviation. Different letters within the mean number of seeds column per population (lower case for Rancho Viejo, upper case for Los Tuxtlas), or fruit set (\*), indicate significant differences ( $p < 0.05$ ) in the number of seeds or fruits within that population. We used 10 individuals for the Los Tuxtlas population, and 14 individuals for Rancho Viejo population.

### Capture of bats and collection of pollen

At Rancho Viejo, one *Anoura geoffroyi* (Fig. 3A) was captured, as well as one *Artibeus toltecus* and five *Carollia sowelli*. Of these, only the *A. geoffroyi* individual presented pollen on the face and wing membrane. Of the pollen carried by this bat, 36.59% was identified as belonging to *P. viridiflora* (through comparison with previously collected samples).



**Figure 3. A)** *Anoura geoffroyi* captured close to a *Pseudalcantarea viridiflora* flower at the Rancho Viejo site (with bright yellow pollen). **B)** *Glossophaga mutica* captured close to a flowering *Pseudalcantarea viridiflora* at Los Tuxtlas. White circles indicate where pollen was found on the body of the bat. Photographs: Pedro A. Aguilar-Rodríguez and M. Cristina MacSwiney G.

At Los Tuxtlas, we captured 72 bats belonging to 11 species and three families during the flowering of this bromeliad: Momoopidae: *Pteronotus mesoamericanus* (1 individual); Phyllostomidae: *Anoura geoffroyi* (1), *Artibeus jamaicensis* (2), *A. toltecus* (36), *Carollia sowelli* (8), *Desmodus rotundus* (7), *Glossophaga mutica* (8), *Hylonycteris underwoodi* (1), *Sturnira hondurensis* (5); Vespertilionidae: *Bauerus dubiaquercus* (2) and *Myotis nigricans* (1). Pollen was found on three of the species listed above: *A. geoffroyi*, *G. mutica* (Fig. 3B) and *H. underwoodi*, all belonging to the nectarivorous Glossophagine subfamily of the Phyllostomidae. Of these, only one *G. mutica* and the sole individual of *H. underwoodi* were not captured close to the flowering *P. viridiflora* individuals, but along flight paths for bats. In the case of *A. geoffroyi*, the pollen was found mainly on the propatagium and plagiopatagium of the wing, while in *G. mutica* and *H. underwoodi*, the pollen was found on the uropatagium. A total of 30.77% of the pollen grains identified on *G. mutica* corresponded to *P. viridiflora* (mean:  $12.62 \pm 18.20$  grains). *Anoura geoffroyi* and the *H. underwoodi* individual also carried pollen of *P. viridiflora*, but only 15 out of 20 grains (75%) and two out of four grains (50%), respectively, were counted.

### Efficiency and observation of floral visitors

The recordings registered six visitor morphotypes: bats, cockroaches, spiders, moths, earwigs, and crickets (Tab. S1

in supplementary material). Considering both nocturnal and diurnal visits, 148 and 160 visits were recorded at Rancho Viejo and Los Tuxtlas, respectively. Of these visitors, only bats were considered to pollinate the bromeliad at both sites, although the frequencies differed. At Rancho Viejo, bats accounted for only 23.65% of the total number of visits, due to a high number of insect visits, while at Los Tuxtlas, bats accounted for 91.25% of all visits. At Rancho Viejo, the most frequently recorded arthropod was cricket sp. 1, with 77 visits (consuming pollen). At Los Tuxtlas, cockroaches (Blattodea) were the most frequently recorded arthropods, with three visits (thieving nectar in all of these visits).

We recorded at Rancho Viejo a total of 35 bat visits to *P. viridiflora* flowers, at a rate of 0.050 visits/flower/hour. At Los Tuxtlas, there were 146 visits, or 0.351 visits/flower/hour. Visits at Rancho Viejo lasted  $0.46 \pm 0.18$  s ( $n = 10$  visits selected at random, range: 0.23-0.73 s), while this was  $1.12 \pm 0.33$  s at Los Tuxtlas ( $n = 10$  visits, range: 0.50-1.40 s). Of the total number of visits by bats, the behavior of the animals indicated a possible pollination on 32 occasions at Rancho Viejo (91.43% of the total number of bat visits; Fig. 4A, Video S1 in supplementary material) and on 100 occasions at Los Tuxtlas (68.49%; Fig. 4B-C, Video S2 in supplementary material). Of these 100 visits at the latter site, the bats hovered in front of the flower on most of the occasions, apart from 11 visits, where these were “perching” on the flower while accessing the nectar. Most of the bat visits at Los Tuxtlas, legitimate or illegitimate (with no contact with reproductive structures), occurred between 19:30 h and 21:00 h, with a peak between 20:00 h and 20:30 h. At Rancho Viejo, the bats recorded visiting *P. viridiflora* did so mainly around 21:00 h, by hovering in each occasion, while no “perching” behavior was observed.

Based on this foraging mode, the size of the bat (relative to the size of the flowers) the observable morphological characteristics (e.g., presence or absence of uropatagium; Fig. 3A-B) and the species captured at the sites, it is most likely that the bats recorded at Rancho Viejo belonged to the species *Anoura geoffroyi*, while at Los Tuxtlas these were either *Glossophaga* sp. or *H. underwoodi*.

The visits at Los Tuxtlas in which, presumably *G. mutica* or *H. underwoodi* perched at the flower to drink its nectar, occurred after the bats arrived and started to hover by the flowers. In these visits, the bats grasped the floral parts (petals, bracts, stamens) with their thumb claws (Fig. 4C), introduced their snouts into the flower calyx and licked the nectar, before finally leaving the flower. This type of visit caused distinct stress to the flower, as it was observed that the stamen and style remained hanging. The calculated relative effectiveness of the visiting bats as pollinators differed between the sites. The relative effectiveness as pollinators of the bat visitors at each site varied; for the bats at Rancho Viejo, the value was 12, while for the bats at Los Tuxtlas, the value was 2.19.

## Intraspecific variability of nectar attracts different bats: the case of *Pseudalcantarea viridiflora*, a bromeliad with crepuscular anthesis



**Figure 4.** Bats visiting *Pseudalcantarea viridiflora* flowers. **A)** Bat, probably *Anoura geoffroyi*, visiting the flower at the Rancho Viejo site. **B)** Smaller bat, probably *Glossophaga mutica*, visiting the flower in flight and “perching” **C)** on the flower. The white circle indicates the position of some anthers during the visit, and the arrow indicates the position of the stigma. Photographs: Pedro A. Aguilar-Rodríguez.

During the diurnal observation period, only arthropod visits were recorded at Los Tuxtlas, while one hummingbird species of the genus *Lampornis* was observed at Rancho Viejo (Tab. S1 in supplementary material). No diurnal visitor appeared to pollinate the flowers of *P. viridiflora* at either site, and the majority of visitors only sought the remnant nectar or pollen in the flowers.

## Discussion

The results show that *P. viridiflora* is a self-compatible bromeliad capable of self-pollination at both sites, and also confirm that the species is pollinated by nectarivorous bats (as reported in Aguilar-Rodríguez *et al.* 2019b). Furthermore, this bromeliad therefore seems to be ecologically specialized (*sensu* Fenster *et al.* 2004) in terms of its pollinator group at either site, since it is primarily chiropterophilous, with

>60% of the visits corresponding to bats (Cruz-Neto *et al.* 2015), although stigmatic receptivity and the presence of pollen on the anthers were observed during diurnal hours following initiation of anthesis.

As expected, there was variation in the bat-pollinators of *P. viridiflora* between the two sites, but it seems that only a subset of the available nectarivorous bat fauna are frequent visitors of this bromeliad. At Rancho Viejo, we found that *P. viridiflora* was pollinated only by *A. geoffroyi*, while at Los Tuxtlas it was pollinated by *G. mutica*, *A. geoffroyi* and possibly also *H. underwoodi* (based on the captured bats as well as analysis of the video recordings). Other potential nectarivorous bats not recorded in this study included *Choeroniscus godmani* for Rancho Viejo, and *C. godmani*, *Glossophaga commissarissi* and *Leptonycteris yerbabuenae* for Los Tuxtlas, while the two sites shared at least *A. geoffroyi* and *G. mutica* (Sosa *et al.* 2008; Coates *et al.* 2017). Thus, other nectarivorous species could potentially pollinate both *P. viridiflora* populations, but these may have been under-represented due to being less tolerant to the anthropized conditions of the study sites (Reid 2009). For example, only one individual of the bat *H. underwoodi* was captured at Los Tuxtlas even after a considerable sampling effort.

Nectar is one of the most important food rewards that contain sugar and other constituents such as amino acids, phenolic compounds or alkaloids that are important for pollinators and serve to influence their preferences and behavior (Parachnowitsch *et al.* 2019; Ryniewicz *et al.* 2020). It is, however, a variable trait: in this study, differences in volume and sugar content were found in nectar traits measured between the two populations of *P. viridiflora* studied, with the highest nectar volume recorded in the Rancho Viejo population. Nectar composition in Bromeliaceae seems to be more influenced by their putative pollinator than by a phylogenetic trait (Krömer *et al.* 2008; Göttinger *et al.* 2019). Even so, nectar composition does not remain constant in individuals of the same species cultivated at different locations (Göttinger *et al.* 2019), or in those individuals cultivated in glasshouses (Canto *et al.* 2007). Different populations of the same flowering species present high variability in terms of sugar composition and nectar volume and concentration (Chalcoff *et al.* 2006; Brown *et al.* 2011; Noe *et al.* 2019). Besides, the volume and sugar concentration of nectar could be altered by endogenous factors, such as individual variability, but also by exogenous factors, such as light, temperature, humidity and others, including the actions of nectar thieves (Boose 1997; Canto *et al.* 2007; Heil 2011) and intra-plant variation (Hodges 1993; Biernaskie & Cartar 2004; Herrera *et al.* 2006). The latter could even exceed the variations found at population level (Zywiec *et al.* 2012; Noe *et al.* 2019).

We recognize that our methods could have affected the nectar measurements obtained in the field and that our samples are relatively limited in number (in part, as a result of the scattered distribution of this bromeliad); however,



in order to compare nectar traits in this manner in the field, we had to contend with many *in situ* variables. For example, it has been identified that nectar traits vary to a greater extent under field conditions than in the greenhouse, probably due to the influence of biotic (e.g., exposure to visitation, presence of yeast in nectar) or environmental (e.g., high humidity) factors (Canto *et al.* 2007; Chalcoff *et al.* 2006; Willmer 2011). Moreover, the action of bagging the flowers in the field may alter nectar volume and concentration (Wyatt *et al.* 1992). We tried to attenuate these environmental effects by taking *ex situ* measurements, but the stress associated with the translocation of the plants, as well as intra-individual variation, might still have had some impact on these measurements.

In some bromeliads, repeated floral visits increase the volume of nectar and the quantity of diluted sugar it contains (Ordano & Ornelas 2004; Stahl *et al.* 2012), but their effects can also be neutral or negative (Willmer *et al.* 2011). The higher presence of nectar-robbers (including hummingbirds) at Rancho Viejo compared to Los Tuxtlas may have also increased the local nectar production (Lara & Ornelas 2002). At this latter site, the higher availability of sympatric flower resources could cause that visiting nectar thieves might ignore the flowers of *P. viridiflora*. Nevertheless, it is interesting to consider that, at Rancho Viejo, where the nectar reward is greater in both volume and sugar concentration, *P. viridiflora* is pollinated by a larger pollinator, *A. geoffroyi*, a species with higher energetic requirements than *G. mutica* (Voigt 2004). Taking into account that *A. geoffroyi* is a common species at Los Tuxtlas, and that it is the main pollinator of other sympatric bromeliads in the study site (i.e., *Pitcairnia recurvata*, Aguilar-Rodríguez *et al.* 2019b), it is unclear why this species does not act as the main pollinator of *P. viridiflora* in this site. Recent studies in Mexican temperate forests show that seasonality affects the sex ratio and phenology of *A. geoffroyi*, promoting regional altitudinal migrations to favor reproduction in this species (Saldaña-Vazquez *et al.* 2020), which might explain its lower abundance at the time when *P. viridiflora* is flowering at Los Tuxtlas. However, further studies are required to explain whether differences in pollinators are directly related to the floral reward, to pollinator population migrations or are produced by specific environmental conditions.

### *Reproductive implications due to different bat pollinators*

Bats are important long-distance pollen vectors (von Helversen & Winter 2003; Fleming *et al.* 2009), transporting even more conspecific pollen from a given plant than hummingbirds (Muchhala & Thomson 2010). Even for a self-compatible plant, a long-distance pollinator could be important for maintaining cross pollination and good progeny quality, especially in fragmented landscapes such as those of this study (Quesada *et al.* 2006; Aguilar *et al.* 2019).

Bat foraging behavior and home ranges vary among species (Ghazoul 2005). A small nectarivorous bat would need to visit fewer flowers and could, in turn, offer shorter pollen transport distances to the bromeliad (see Hmeljevski *et al.* 2017) than a larger bat. The home range of *G. soricina* is small (Fleming *et al.* 1993), with a flight distance of at least 500 m and up to 3-5 km (Aguilar *et al.* 2014). This represents a smaller area than that utilized by larger nectarivorous bats that are more mobile and can track resources over larger areas, such as *Leptonycteris* spp. (von Helversen & Winter 2003). Based on its flight morphology and size, the larger *A. geoffroyi* could be expected to utilize a larger home range than *G. mutica* (Sperr *et al.* 2011) or *H. underwoodi* and could thus serve as a long-distance pollinator for the bromeliad.

Even if not statistically significant (probably because of the uneven number of treatments at both sites), it seems that when we exclude the possibility of self-pollination in the field, the fruit set and seed set both decrease. It is important to note that self-pollination, which takes place prior to exposure of the flowers to pollinators (such as in the case of *P. viridiflora*), acts to reduce the number of ovules available for conspecific pollen from another individual, but guarantees a certain number of fertilized ovules (Lloyd 1992). Such a reproductive system that ensures self-pollination, or performs it to an intermediate extent, as would seem to be the case in *P. viridiflora* at both sites, can be an advantageous backup-system when pollen is limited in the habitat (Kennedy & Elle 2008).

Different bat species could affect the reproductive output of flowering plants through differences in their behavior, e.g., in the mode and frequency of visits, as well as in body mass (Tschapka 2003; Frick *et al.* 2013; Stewart & Dudash 2017a). Furthermore, angiosperm species that share pollinating bats have been reported to deposit/collect pollen on various body parts of the bat, thus facilitating reproductive isolation (Muchhala & Thomson 2012; Stewart & Dudash 2016), although we still lack sufficient information in this regard and cannot rule out the possibility that *A. geoffroyi* and *G. mutica*/*H. underwoodi* might be functionally complementary to the bromeliad (Fründ *et al.* 2013).

It should be noted that the relatively long style of the other two self-compatible but strictly xenogamous species of *Pseudalcantarea* (>12 mm in *P. grandis* and *P. macropetala*; Krömer *et al.* 2012) might be of sufficient length to prevent pollination by smaller bats, such as *G. mutica*. This could broadly suggest a tendency towards evolutionary specialization in “larger” bat species and less reliance on spontaneous self-pollination for this genus. For example, *A. geoffroyi* is the main pollinator recorded for *P. macropetala* in a population near to the one studied here at Rancho Viejo (Aguilar-Rodríguez *et al.* 2014). While visiting this species, *A. geoffroyi* presented a similar visitation frequency (0.034 visits/flower/hour) as recorded for *P. viridiflora* at Rancho Viejo (0.050 visits/flower/hour), obtaining in both cases a PLI about zero. This contributes



to the notion that, even if it is as frequent a pollinator as members of *Glossophaga* spp., this larger bat species might transport enough pollen to be an advantageous pollinator to other bigger and xenogamous bromeliads that present a poor fit between their reproductive organs and the bodies of smaller nectarivorous bats, even if the floral morphology is almost identical to that of *P. viridiflora*.

Pollen load was more conspicuous on the forehead fur and wings of the *A. geoffroyi* captured at both sites than on the uropatagium of the *G. mutica* captured at Los Tuxtlas (Fig. 3A-B). The stigma of *P. viridiflora* should be able to collect pollen from the body of the bat, but it is likely that pollen in the fur of *A. geoffroyi* (on its forehead and arms) will have a greater opportunity for deposition on the stigma than that found on the small and less hairy uropatagium of *G. mutica*. We acknowledge that some pollen on the wings may be lost during manipulation of the bats, even though we tried to collect it as soon as possible before releasing the bat from the net to take measurements. The percentage of bromeliad pollen carried by the bats is only a proxy for the actual proportion of the presence of the bromeliad in the bat's diet, but it is useful to understand that, in addition to *P. viridiflora*, both *A. geoffroyi* and *G. mutica* also visit other plant species during the flowering period of this bromeliad (see other examples in Sánchez-Casas & Álvarez 2000; Caballero-Martínez *et al.* 2009; Aguilar-Rodríguez *et al.* 2019b).

Visits by smaller nectarivorous bats in Los Tuxtlas (either *G. mutica* or *H. underwoodi*), were longer in duration than those of *A. geoffroyi* ( $1.12 \pm 0.33$  s vs.  $0.46 \pm 0.18$  s respectively), and the rate of visits was also higher at Los Tuxtlas than in *A. geoffroyi* at Rancho Viejo ( $0.351$  vs.  $0.050$  visits/flower/hour). A total of 91 % of the visits of *A. geoffroyi* were legitimate, compared to 68 % of *G. mutica*. Relative effectiveness as a pollinator of *P. viridiflora* in both bat species differs widely, considering the rate of visits and fructification derived exclusively from the pollen transported by these pollinators. While the smaller bats at Los Tuxtlas present a higher visit rate, *A. geoffroyi* requires fewer visits to achieve the same reproductive success seen in the field (bearing in mind that part of this fructification is derived from the pollen of *P. viridiflora* itself, because of spontaneous pollination). It would be valuable to conduct *ex situ* experiments to assess whether either of these bats actually sires more seeds per visit. The most widely accepted method to measure effectivity in pollination ecology is comparison of per-visit fruit set among pollinator taxa (Ne'eman *et al.* 2010; King *et al.* 2013); however, this method is effective only for those plants and pollinator taxa for which a visit is necessary in order to begin fruit formation (Wang *et al.* 2017) and is therefore inappropriate for species with multiple ovules (Ne'eman *et al.* 2010). Moreover, it is methodologically complicated to accurately document per-visit effectivity in nocturnal floral visitors.

In conclusion, while both the reproductive system and pollination mode of *P. viridiflora* appear to be similar in both populations, there are some interesting differences. First, the fruit set and seed set of the treatments at Los Tuxtlas are affected by the site, possibly due to an environmental factor, which could also include those pollinators that help the flowers to reach a higher fruit and seed set such as that of the Control or Xenogamy treatments. Secondly, the nectarivorous bat species recorded as putative pollinators are of different species, which also differ in terms of their visitation behavior and size, both of which are traits that could produce a differential contribution to pollination of the bromeliad. Thirdly, we found at least an indication of differences in nectar production between sites, which could, in theory, favor the maintenance of a larger bat pollinator.

Knowledge of the natural history of a plant-animal interaction is the first step towards addressing more complex questions regarding the nature of this interaction and determining how different species could contribute to the reproduction of a plant. Pollinator size and the frequency and manner in which the pollinators undertake floral visits, could promote differences in the contributions of sympatric pollinating species to the reproductive success of the visited plants. It is also worth noting that pollinator effectiveness can change according to season (Fishbein & Venable 1996; Rocca & Sazima 2013; Leal *et al.* 2020). Further and more detailed studies should use other indicators of reproductive success (*i.e.*, germination and vigor test in the seeds or other post-seed variables or genetic components: Valverde *et al.* 2019) with an *ex situ* experimental design, in order to assess pollinator effectiveness among members of the same pollinator guild.

## Acknowledgements

We thank José Luis Libreros, Esteban Francisco Ventura, Oscar Ameca, Pedro Díaz, Emmanuel Pantoja, Zuemy Vallado, Lilia Ruiz, Hebert P. Hernández, Emmanuel Solís, Misael Nieto, Samaria Armenta, Luis Abarca, Enriqueta Velarde and the community group Los Clarines. We also thank Andrew P. Vovides and Sonia Galicia from INECOL for help in the laboratory. This work was supported through a doctoral grant to PAAR from CONACYT (No. 59406). Keith MacMillan and Erick Wild corrected the English version of the manuscript.

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## Intraspecific variability of nectar attracts different bats: the case of *Pseudalcantarea viridiflora*, a bromeliad with crepuscular anthesis

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## Intraspecific variability of nectar attracts different bats: the case of *Pseudalcantarea viridiflora*, a bromeliad with crepuscular anthesis

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