Acta Botanica Brasilica - 34(1): 177-184. January-March 2020.
doi: 10.1590/0102-33062019abb0219

Pollination biology of the endangered columnar cactus *Cipocereus crassisepalus*: a case of close relationship between plant and pollinator

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Received: June 26, 2019
Accepted: October 22, 2019

ABSTRACT

The family Cactaceae possesses considerable floral diversity and is exclusively zoophilic. *Cipocereus crassisepalus* is an endangered species of columnar cactus, whose floral characteristics fit the chiropterophily syndrome. This study aimed to assess the correspondence between floral traits and pollinators and whether the pollination system of *C. crassisepalus* is specialized, as suggested by the hypothesis of geographical dichotomy. Hand pollination treatments demonstrated that *C. crassisepalus* depends on cross-pollen flow mediated by pollinators to set fruit and seeds. Nocturnal film recordings, diurnal observations, analyses of pollen loads of flower visitors and visitor exclusion experiments provided strong evidence that bats are the sole pollinators of this cactus. During two flowering seasons, pollen grains of *C. crassisepalus* were found only on the bodies of the bat *Anoura caudifer*. *Cipocereus crassisepalus* possesses a pollination system specialized on nectar-feeding bats, which corroborates the geographical dichotomy hypothesis.

Keywords: *Anoura caudifer*, Cactaceae, Chiropterophily, effective pollinator, self-incompatibility

Introduction

The family Cactaceae, with over 1,450 species in 127 genera (Hunt *et al* . 2006), occurs in a wide variety of temperate and tropical environments throughout the Americas, including extremes such as the Atacama Desert and flooded Amazon forests (Schlumpberger 2012), which consequently involves species with different habits (Grant *et al* . 1979; Anderson 2001). Cacti also show considerable floral diversity, with differences in form, size, anthesis time and resources (Faegri & Pijl 1979; Grant *et al* . 1979). All of the main pollination syndromes are represented, *i.e.*, melittophily, ornithophily, sphingophily and chiropterophily (Faegri & Pijl 1979). Cactus flowers are exclusively zoophilic (Grant *et al* . 1979; Anderson 2001) and most species are obligate out-crossers, dependent on animal pollination services for reproduction (Ross 1981; Boyle 1997; Mandujano *et al* . 2010).

Several species of columnar cacti are dependent to some degree on bats and birds for pollination (Bustamante *et al* . 2016). These cacti show phenotypic and functional specialization (*sensu* Ollerton *et al* . 2007) in their interactions with their vertebrate pollinators. Some species may possess mixed pollination systems, *i.e.*, functional and ecological generalization (*e.g.* Fleming *et al* . 1996; 2001), although they show phenotypic specialization. The existence of these specializations has suggested the geographic dichotomy hypothesis, referring to columnar cacti and their pollination systems (see Valiente-Banuet *et al* . 1996). This hypothesis suggests that specialized pollination systems involving bats and columnar cacti would be centered in tropical areas (Valiente-Banuet *et al* . 1996; 1997a; b; Nassar *et al* . 1997), with a progressive reduction in the degree of specialization

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toward extra-tropical regions, where diurnal animals would also serve as pollinators (Fleming *et al* . 1996; 2001).

Although several studies have supported the geographic dichotomy hypothesis in columnar cacti, most of them were conducted in the northern hemisphere and little information is available on the pollination systems of cacti in the southern hemisphere (Arzabe *et al* . 2018). Brazil, for example, is considered the third-largest center of cactus diversity (Taylor 1997), with high rates of endemism (Ribeiro-Silva *et al* . 2011), but pollination modes, not only of columnar cacti but of the family as a whole, are little known. Studies of reproductive and floral biology of cacti have examined only about 5% of the species recorded for the country (Teixeira *et al* . 2018).

Columnar cacti are a highly diverse group of plants, with at least 170 species in four tribes within the subfamily Cactoideae (Browningia, Cereeae, Pachycereeae and Trichocereeae) (Fleming & Valiente-Banuet 2002). The majority of columnar cacti show specializations that indicate pollination by nectar-feeding bats (Nassar *et al* . 1997; Fleming *et al* . 2001; 2009; Valiente-Banuet *et al* . 2004; Munguía-Rosas *et al* . 2009). *Cipocereus crassisepalus* is a rare columnar cactus species endemic to the Espinhaço Mountain Range region of southeast Brazil. Although this species is listed as "endangered" by the IUCN, mainly because of habit destruction (Zappi & Taylor 2013), its reproductive biology is unknown. The flowers of *C. crassisepalus* are nocturnal, white, large, tubular and robust, typical attributes of chiropterophily. This study documented the breeding system, floral biology, pollination system and pollinator effectiveness of this cactus. In addition, we evaluated the correspondence between floral traits and the pollination system. We expected that according to the geographical dichotomy hypothesis, *C. crassisepalus* would show a pollination system specialized for bats.

Materials and methods

Study site and species

We studied one population of *Cipocereus crassisepalus* (Buining & Brederoo) Zappi & Taylor, from July 2011 to December 2012, in the Parque Estadual do Rio Preto (PERP), a protected area (18°07'12.9"S 43°20'36.9"W) in the municipality of São Gonçalo do Rio Preto, Minas Gerais, Brazil. The PERP is located in the Espinhaço Mountain Range and possesses a mosaic of Cerrado physiognomies. We conducted the study in an area dominated by cerrado *sensu stricto*, riparian forests, and "campos rupestres", which occur on different substrates along an elevation gradient from 950 to 1000 m a.s.l. This population of *C. crassisepalus* grows on patches of quartzitic sandy soil, sometimes bordered by tangled, shrubby vegetation (Fig. 1A-B). The climate is characterized by a distinct rainy season from November to March and a dry season from June to September. The mean annual temperature is 18.9 °C (Brasil 2004). A voucher specimen of *C. crassisepalus* was deposited in the herbarium RB (786543).

Cipocereus crassisepalus belongs to tribe Cereeae, a dominant group in eastern Brazil (Taylor & Zappi 2004; Zappi *et al* . 2010). The genus *Cipocereus* comprises six described species (Zappi *et al* . 2010). Individuals of *C. crassisepalus* can reach up to 3 m in height and have a poorly branched green stem bearing 4–6 ribs (Fig. 1B). The species is endemic to the state of Minas Gerais, restricted to the Espinhaço Mountain Range, and occurs solely on deposits of quartzitic sand (Taylor & Zappi 2004).

Floral traits

We recorded the time of anthesis and, using digital calipers, we measured 20 newly opened flowers from 20 individual plants. Flower measurements included the corolla diameter, flower length, floral-tube length (Fig. 1C), and number of anthers and ovules. Stigma receptivity was determined for 10 flowers of 10 individual plants by using 10 % hydrogen peroxide during the first hours of anthesis (18h00min and 20h00min). We measured the volume of accumulated nectar during the morning (8h00min – 9h30min) of 25 bagged flowers of 15 individuals. Nectar volume was measured using microliter syringes (100 µL; Hamilton, U.S.A.), and nectar sugar concentration was quantified with a pocket refractometer (Atago® N1, Brix scale 0–32 %).

Controlled-pollination and visitor-exclusion experiments

To determine the breeding and mating system of *C. crassisepalus,* in July 2011 we performed an experiment with four treatments: (1) autonomous self-pollination – flowers in pre-anthesis were kept bagged, with no additional manipulation; (2) hand self-pollination – flowers were hand-pollinated with their own pollen; (3) nocturnal hand cross-pollination – flowers were emasculated and pollinated with pollen from at least two flowers of different individuals 10 m apart from each other; and (4) natural pollination – flowers accessible to pollinators were individually marked (control). Hand treatments were conducted between 19h30min and 21h00min, when the stigma was receptive and anthers were releasing pollen (Dafni *et al* . 1992). To determine if the flowers set fruits when pollinated during the day, in July 2011 we performed diurnal hand cross-pollination: flowers were emasculated and pollinated with pollen grains from at least two flowers of different individuals 10 m apart from each other, between 8h30min and 10h00min. Except for the control, we bagged all the flowers before applying the pollination treatments. After treatment, all flowers remained bagged until fruit formation or flower abortion. For pollination treatments, we used 20 to 45 flowers per treatment, from 30 individuals. The production of fruits

and seeds was evaluated for each treatment, to determine the predominant reproductive system.

To evaluate the effectiveness of diurnal and nocturnal pollinators, in July 2012 we conducted exclusion experiments during five consecutive days and nights. Thirty-three flowers of 20 individuals were bagged to exclude diurnal visitors from 5h30min until the beginning of anthesis (nocturnal pollination), and 33 flowers of 20 individuals were bagged to exclude nocturnal visitors from 17h30min to 5h30min of the next morning (diurnal pollination). Fruit and seed production was assessed for each treatment, to evaluate the relative contributions of different groups of visitors to reproductive success. The evaluation of the effectiveness of diurnal and nocturnal pollinators considered the total contribution by each type of pollinator to reproductive success (Freitas 2013).

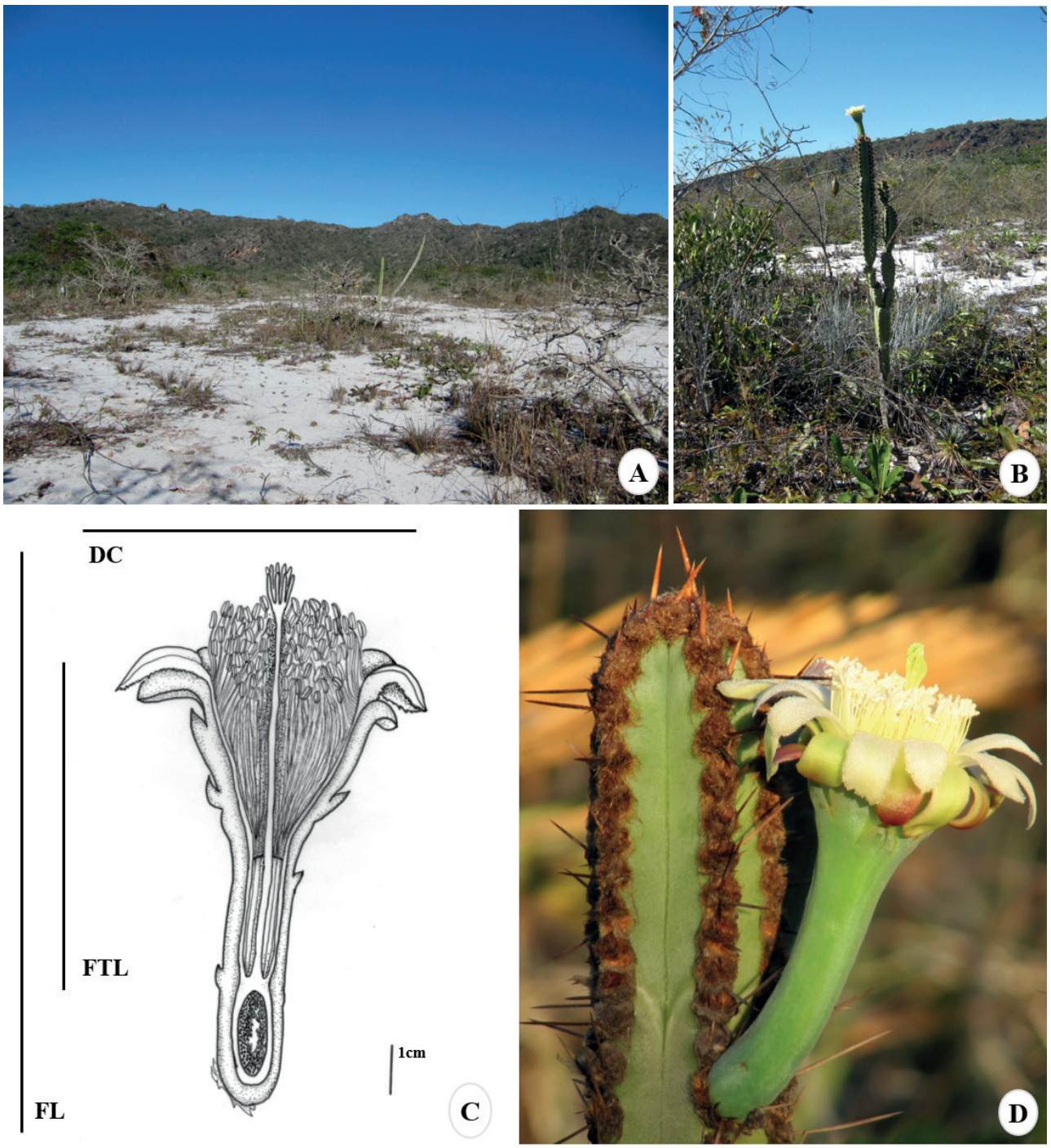

Figure 1. Population studied and individual of *C. crassisepalus* in the Parque Estadual do Rio Preto (**A-B**). Flower of *Cipocereus crassisepalus*; some flower's measurements, diameter of the corolla (DC), flower length (FL), floral tube length (FTL) (**C**), flower during anthesis (**D**) (Design: Viviane Scalon).

Floral visitors and pollinators

To identify nocturnal visitors, we captured bats with mist nets, and hawkmoths and nocturnal bees with black-light traps, and analyzed the pollen grains adhered to their bodies. Three mist nets measuring 10.0 x 2.5 m were opened for 19 consecutive days in July (8550 h.m^2) and four days in December 2012 (1800 h.m^2). Three black-light traps were installed during two successive nights in July 2012 (24 trap-hours). Both the mist nets and the black-light traps were installed among individuals of *C. crassisepalus*. Pollen grains adhered to the face and chest of bats, to the body of bees, and to the tongue of hawkmoths were removed by pressing small pieces of glycerin gelatin colored with fuchsin against the body of the animals (Beattie 1971; Louveaux *et al*. 1978). For each animal that showed pollen on its body, a slide of the pollen was prepared, and later this slide was compared to a reference slide made with the pollen of *C. crassisepalus*.

Since the pollen grains of species of *Cipocereus* are indistinguishable under an optical microscope, we were not able to distinguish between the pollen of *C. crassisepalus* and *C. minensis* (sympatric in the area; Martins *et al*. 2016) taken from the flower visitors captured during the flowering period in the dry season, in July 2012. However, we believe that no pollen of *C. minensis* was present, because the mist nets were purposely placed between individuals of *C. crassisepalus* in a sandy region where there were no populations of *C. minensis*.

To check for the presence of nocturnal visitors and characterize the behavior of bats during flower visits, we videotaped one focal flower with a Sony HDR-XR160 digital camcorder. The camera was placed on a tripod circa 1.5 m distant from the plant. Video recordings were made from 20h30min to 22:h30min on four consecutive nights in December 2012, totaling eight hours of recording. We recorded the type of visitor (bat or insect), number and duration of visits, and if the visitor contacted the stigma.

We conducted daytime observations from 6h00min to 10h30min for four days, totaling 27 hours of sampling, in July 2012. The frequency of diurnal floral visitors was determined by recording the number of visits during 20 min of observation for each flower, one flower per individual. Observations were made by an observer, who remained approximately 1 m distant from the plant. When possible, we collected samples of flower visitors for identification, except for *Apis mellifera*, for which only the number of visits was recorded. Daytime observations of pollinators were not carried out during the rainy season (December 2012); during that period, the flowers were already closed at dawn.

Statistical analysis

We constructed generalized linear mixed models (GLMM), with Poisson error distribution and using the glmer function, to evaluate differences in seed set in nocturnal hand cross-pollination and natural pollination. The number of seeds was included as dependent variable, pollination treatments as a fixed effect, and plant as a random factor. To compare the pollination efficiency of nocturnal and diurnal flower visitors, we ran another GLMM, with Poisson error distribution and using the glmer function, with the number of seeds as dependent variable, visitor exclusion period (nocturnal and diurnal) as a fixed factor, and plant as a random factor. All statistical analyses were performed using R software version 3.3.2 (R Development Core Team 2017).

Results

Floral traits

Cipocereus crassisepalus flowers twice a year, during the dry season (July and August) and again in the rainy season (December and January); during the dry season, its flowering overlaps with that of its sympatric congener *Cipocereus minensis* subsp. *leiocarpus*. The flowers of *C. crassisepalus* are hermaphroditic, large, robust, and tubular, with a corolla diameter of 5.59 ± 0.66 cm (mean ± SD throughout the text). The flower and flower-tube lengths were 9.29 ± 1.09 and 5.57 ± 0.54, respectively. Approximately 500 stamens (468.95 ± 59.53) of different heights are placed around the style. The number of ovules per flower was 2023 ± 508.72 (Fig. 1 C-D).

Anthesis starts at around 17h00min, with slow opening of the outer and inner perianth segments. The stamens are initially curved toward the center, with introrse anthers around the style, and become erect with advancing anthesis. The flowers reach their maximum opening at 20h30min and the stigma is already receptive at 19h30min. The flowers begin to close early on the next day, at 9h00min, and by 11h00min to 11h30min all the flowers are closed. Hence, the floral anthesis of *C. crassisepalus* lasts approximately 16 h in the dry season. In the rainy season the flowers were already closed before dawn. *C. crassisepalus* flowers produce a sweet odor and abundant, relatively dilute nectar. The mean volume of nectar accumulated by the flowers was 1.03 ± 0.38 mL (0.10 – 1.64 mL; N = 25) and the mean concentration was 17.45 ± 2.2 % (14 – 22 %; N = 25).

Controlled pollination and visitor exclusion experiments

Autonomous and self-pollinated flowers did not set fruit. Fruit formation occurred only in flowers that underwent hand cross-pollination and natural pollination (control). Seed production in nocturnal hand cross-pollination was significantly higher than natural pollination (X^2 = 2524.2; df = 1; N = 71; p < 0.001; variance random effect = – 17.02; SD = 4.126). Because autonomous and hand self-pollination set no fruit, we did not include these treatments in the model. The results of the diurnal hand cross-pollination treatment showed that the flowers of *C. crassisepalus* are

still receptive at the end of anthesis, *i.e.*, they retain their reproductive potential while they are open (N = 20 flowers, fruit set = 75%, seed number = 1150.33 ± 497.14). Flowers that were available only to nocturnal visitors produced significantly more seeds than those available only to daytime visitors (X^2 = 2067.0; df = 1; N = 70; p < 0.001; variance random effect = 39.69; SD = 6.3) (Tab. 1).

Floral visitors and pollinators

We captured 45 bats representing 11 species (34 in July, in the dry season; and 11 in December, in the rainy season). We collected pollen of *C. crassisepalus* from 11 of 21 nectarivorous bat specimens captured. All bats with pollen of *C. crassisepalus* belonged to *Anoura caudifer* (subfamily Glossophaginae) (Fig. 2A). Three species of glossophagine bats, *Anoura geoffroyi, Glossophaga soricina* and *Lonchophylla dekeyseri*, had no pollen of *C. crassisepalus*. Individuals of *A. caudifer* possessed pollen of other, unidentified plant species in addition to *C. crassisepalus* pollen. Unidentified pollen types were also found on the non-nectarivorous bats *Micronycteris megalotis*, *Platyrrhinus lineatus* and *Dermanura* sp. (Tab. 2). Pollen grains of *C. crassisepalus* were also not found on the individuals of hawkmoths (n = 4) (Lepidoptera, Sphingidae) and on the individuals of *Megalopta* sp. bees (n = 7) (Hymenoptera, Halictidae) caught in a light trap.

On average, 5 ± 2.65 (mean ± SE) bats per night (2 h/night) visited the flowers of *C. crassisepalus*. Visits were short, lasting less than a second, and the bats hovered over the flowers while imbibing nectar. When visiting a flower, bats inserted their head into the flower and contacted the anthers and stigma with their rostrum, head, chest or throat, depending on the angle of approach. We recorded a single visit of an unidentified species of Sphingidae; thus, this visitor was not considered an important pollinator for *C. crassisepalus*.

Diurnal flower visitors were the bees *Apis mellifera*, *Trigona spinipes*, *Trigona hyalinata, Oxytrigona tataira,* and species of Halictidae. We recorded only one visit by the hummingbird *Eupetomena macroura* (Trochilidae). The most frequent diurnal visitor species was *Apis mellifera*. During the day, *A. mellifera* was the first to visit the flowers, at around 7h00min. The bees remained active for one hour and then the number of visits began to drop. When the numbers of *A. mellifera* became large, we observed some contact with the stigma. As the numbers of *A. mellifera* decreased, *Trigona* and some individuals of Halictidae species arrived at the end of anthesis. The contact of the bees with the reproductive parts of the flowers consisted solely of movements between the stamens, for pollen collection (Fig. 2B).

Table 1. Fruit set and seed number in each pollination treatment on flowers of *Cipocereus crassisepalus*.

Pollination treatments	Flowers (n)	Fruit (n)	Fruit set (%)	seeds/fruit (Mean±SD)
Autonomous self-pollination	40	0	-	-
Hand self-pollination	31	0	-	-
Nocturnal hand cross-pollination	30	27	90	1591.81 ± 523.83
Diurnal hand cross-pollination	20	15	75	1150.33 ± 497.14
Natural pollination (control)	45	19	42	1114.22 ± 656.81
Nocturnal pollination	35	23	66	1432.17 ± 605.68
Diurnal pollination	35	2	6	143.50 ± 17.68

Table 2. Species of bats sampled in the study area, their respective abundances (N), number of pollen types adhered to their bodies and indication of the presence (X) or absence (-) of pollen of *Cipocereus*.

Bat species	N	Pollen types (n)	With pollen of *C. crassisepalus*
Glossophaginae			
Anoura caudifer (E. Geoffroy, 1818)	12	14	X
Anoura geoffroyi (Gray, 1838)	2	1	-
Glossophaga soricina (Pallas, 1766)	3	7	-
Lonchophylla dekeyseri (Taddei, Vizotto & Sazima, 1983)	4	2	-
Stenodermatinae			
Artibeus obscurus (Schinz, 1821)	1	0	-
Artibeus (Artibeus) sp.	2	0	-
Artibeus (Dermanura) sp.	8	1	-
Platyrrhinus cf lineatus (E.geoffroyi,1810)	9	3	-
Platyrrhinus sp.	1	0	-
Carolliinae			
Carollia cf perspicillata (Linnaeus, 1758)	1	0	-
Phyllostominae			
Micronycteris megalotis (Gray, 1842)	2	1	-

Figure 2. *Anoura caudifer* the effective pollinator of *C. crassisepalus* (**A**) and *Apis mellifera* visiting the flowers of this cactus species (**B**).

Discussion

Cipocereus crassisepalus is a xenogamic species with all the floral characteristics of chiropterophily pollination syndrome, dependent on nectarivorous bats for fruit and seed production. During two flowering seasons, the tailless bat *A. caudifer* was the only nectarivorous bat showing pollen grains of *C. crassisepalus* on its body surface, despite the presence of three other species of nectarivorous bats in the study area. *Cipocereus crassisepalus,* similarly to *C. minenis* (Martins *et al* . 2016) and *C. laniflorus* (Rego *et al* . 2012), possesses chiropterophilous flowers (Faegri & Pijl 1979) with phenotypic and functional specialization (*sensu* Ollerton *et al* . 2007). These results are in accordance with the geographic dichotomy hypothesis, in which columnar cacti in the tropics will possess pollination systems specialized for nectar-feeding bats (see Valiente-Banuet *et al* . 1996). This is the first study to show *A. caudifer* as a pollinator of columnar cacti in Brazil, although other glossophagine bats are known to pollinate *Pilosocereus catingicola* (*G. soricina*; Locatelli *et al* . 1997), *Micranthocereus purpureus* (*L. mordax*; Aona *et al* . 2006); *Pilosocereus tuberculatus* (*G. soricina* and *L. mordax*; Rocha *et al* . 2007) and *Cipocereus laniflorus* (*G. soricina* and *A. geoffroyi*; Rego *et al* . 2012).

The controlled pollination experiments showed the probable presence of a self-incompatibility system for *C. crassisepalus* and dependence on pollinators for fruit and seed set. Self-incompatibility is widespread among columnar cacti (Mandujano *et al* . 2010). The species *C. laniflorus* (Rego *et al* . 2012) and *C. minensis* (Martins *et al*. 2016) do not produce fruit by self-pollination (*i.e.* 50% of the genera are xenogamic), and hand pollination in two different populations of *C. minensis* subsp. *leiocarpus* reinforced the hypothesis of self-incompatibility as an attribute of the genus.

The presence of *C. crassisepalus* pollen grains only on *A. caudifer,* despite the occurrence of other nectarivorous bats in the local assemblage, suggests an ecological specialization of the pollination system of this cactus (*sensu* Gomez & Zamorra 2006; Ollerton *et al* . 2007). Patterns of interaction between plants and pollinators are determined by aspects such as species abundance, their spatio-temporal distributions, and especially the length of the corolla and the pollinator mouthparts (Stang *et al* . 2006; Olesen *et al* . 2011; Vizentin-Bugoni *et al* . 2014). The number of flower-visiting species should decrease with increasing depth and decreasing width of floral tubes (Stang *et al* . 2006). Nectarivorous bats differ not only in the length of the rostrum, but also in the extent of their tongues, so that the maximum tongue extension is tightly correlated with the length of the rostral components (Muchhala 2006). In a study of *Centropogon nigricans*, the authors reported that the species was pollinated by three species of bats, although other species also occurred in the study area. According to the authors, the different

species were not able to reach the nectar in the long floral tubes of *C. nigricans* (*see* Muchhala 2006). We believe that a similar phenomenon may occur with *C. crassisepalus*. The large size of the floral tube of *C. crassisepalus* may act as a filter, excluding other potential, but less effective pollinators.

The production of fruits from diurnal hand-pollination indicates that the stigmas of flowers of *C. crassisepalus* are still receptive during the morning, and that fertilization is possible. During the day, flowers are visited by hummingbirds and bees. However, diurnal visitors contribute 11-fold less than nocturnal flower visitors to fruit set. Moreover, flowers visited exclusively during the day set fruits containing half the number of seeds of those visited only by nocturnal pollinators, similar to observations of other plants with predominantly nocturnal anthesis (see Ibarra-Cerdeña *et al* . 2005; Munguía-Rosas *et al* . 2010; Ortega-Baes *et al* . 2011). *Apis mellifera* was the most common daytime visitor, but its small size and behavior did not favor adequate pollination of flowers, as observed by other authors for other species of cacti (Rivera-Marchand & Ackerman 2006; Rocha *et al* . 2007; Ortega-Baes *et al* . 2011). The other bees observed did not perform legitimate visits, were present in low numbers, and behaved as opportunistic visitors. For example, *Trigona* sp. arrived at the end of anthesis and caused damage to the flowers, cutting anthers and stigmas.

In short, *C. crassisepalus* is self-incompatible, with a specialized pollination mode that depends on bats for fruit and seed production, as suggested by its floral characteristics, which are consistent with the syndrome of chiropterophily. In order to develop conservation strategies for endangered plant species, it is necessary to understand certain aspects of their natural history, such as reproductive biology. In addition, we should understand the role of the interactions of plant species with animals, especially pollinators and seed dispersers. Knowledge of the reproductive biology of *C. crassisepalus* provides support for actions to conserve this rare, endemic and endangered species of cactus.

Acknowledgements

We are grateful to the following institutions for providing scholarships: Conselho Nacional de Pesquisa (CNPq) , Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (CAPES/PNPD-1432299). FAPEMIG (APQ-00907-12) and CAPES-PRODOC supported the study. We thank the Instituto Estadual de Florestas (IEF) for permission to work in the nature reserve and the director of the reserve, Antônio Almeida Tonhão and the staff of Parque Estadual do Rio Preto, for logistic support. We thank Viviane Scalon for floral design.

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