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Original article

Who are the pollinators of *Petunia interior* (Solanaceae) and how are they attracted to flowers?

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ABSTRACT

Many features of flowers comprise the key elements of the pollinating strategies of flowering plants. Our aims were to describe features that attract pollinators and to identify the pollination system of *Petunia interior,* a species for which bees have been suggested as the probable pollinators. Therefore, we described the morphology and floral biology, assessed nectar production, concentration, and composition, examined reproductive mode and identified pollinators. *P. interior* has a purple, infundibuliform, zygomorphic corolla with a short and wide tube and blue pollen. Flower opening and pollen release were asynchronous throughout the day. The pollen grains have pollenkitt on the surface. The nectar sugar composition has a proportion of sucrose lower than the proportion of glucose + fructose, and the nectar supply was constant, in small amounts, at a concentration between 16.6-23.1 %. The reproductive system is xenogamous and bees were the exclusive pollinators. *P. interior* exhibits a set of floral traits that prevent self-pollination and maintains attractiveness to the bees. The greater reproductive success under natural conditions highlights the importance of bees for the reproductive success of *P. interior.* As far as floral traits are concerned, only the sugar concentration in the nectar does not correspond to melittophily.

Keywords: floral traits, melittophily, nectar composition, oligolectic bees, pollination biology, reproductive biology

Introduction

Plants adopt a number of different strategies to avoid self-pollination, attract floral visitors and promote cross pollination (Barret 2003; Ollerton *et al*. 2011). Floral resources, for example, are made available to attract and reward potential pollinators (Faegri & Van Der Pjil 1971). Plants also have mechanisms to optimize the frequency and behavior of flower visitors, such as increasing floral display (Mitchell *et al*. 2004; Karron & Mitchell 2012) or by gradually supplying pollinators with floral resources (Willmer & Stone 2004; Siriani-Oliveira *et al*. 2018; Araújo *et al*. 2019). Finally, many plants may evolve a set of floral traits that are responsible for attracting specific groups of pollinators, which in turn transport and deposit conspecific pollen in the stigma efficiently (Faegri & Van Der Pjil 1971).

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Petunia (Solanaceae) is a genus with hermaphroditic flowers, consisting of 14 endemic species from subtropical and temperate areas of South America, whose center of origin occurs in southern Brazil (Stehmann *et al*. 2009). These species exhibit flowers with different shapes and colors adapted to specific pollinator groups: *P. axillaris* has white flowers pollinated by nocturnal moths (Ando *et al*. 2001; Hoballah *et al*. 2007); *P. exserta* has red flowers pollinated by hummingbirds (Lorenz-Lemke *et al*. 2006; Stehmann *et al*. 2009); *P. secreta* has traits attractive to several functional pollinator groups and appears to follow an evolutionary transition in its pollination system (Rodrigues *et al*. 2018); and *P. integrifolia* (Wittmann *et al*. 1990; Ando *et al*. 2001; Castellani & Lopes 2002) and *P. mantiqueirensis* (Araújo *et al*. 2019) are pollinated by bees. For the other species of the genus, bee-pollination is predicted (Stehmann *et al*. 2009).

Floral traits such corollas with a wide and short tube and purple UV-reflecting, androecium inserted into the corolla tube, blue pollen, nectar produced in smaller volumes and higher concentrations, with a predominance of glucose and fructose sugars and diurnal flower opening are among the floral traits recorded in bee-adapted petunias (Wittmann *et al*. 1990; Ando *et al*. 2001; Castellani & Lopes 2002; Brandenburg *et al*. 2012; Araújo *et al*. 2019). Therefore, all petunias with these characteristics are expected to have bees as their exclusive or main pollinators (Stehmann *et al*. 2009; Gübitz *et al* 2009).

Although *Petunia* has become a model for studies on how floral diversification was driven by different pollinators (Gübitz *et al*. 2009; Fregonezi *et al*. 2013; Rodrigues *et al*. 2018), the literature with data on floral biology and the relationship with pollinators in natural conditions remains scarce. In this context, we assessed the floral biology, nectar secretion and composition, reproductive biology, and also made field observations for flower visitors and recorded the pollinators of *P. interior*, a species belonging to the group of purple petunias, whose bee pollination syndrome is predicted (Stehmann *et al*. 2009; Reck-kortmann *et al*. 2014). Our goals were: (i) to describe pollinator-attraction traits, and (ii) to understand this species' pollination system. Our questions were as follows: (i) How can floral attributes affect pollinator attraction and the reproduction of *P. interior*? and (ii) Is there an association between floral traits and the predicted pollination syndrome?

Materials and Methods

Species occurrence and Study site

The distribution of *Petunia interior* Ando & Hashim. is restricted to some points in the western region of Santa Catarina, north and northwest regions of Rio Grande do Sul, and also in the province of Misiones (Argentina) (Ando & Hashimoto 1996; Ando *et al*. 2005).

We conducted this study in 2018 and 2019, from September to December, in an area belonging to the Federal University of Fronteira Sul (28°08'29.5" S; 54°45'42.2" W) in the city of Cerro Largo, state of Rio Grande do Sul, Brazil. In this location there are natural populations of *P. interior*. The vegetation of Cerro Largo encompasses Seasonal Deciduous Forests (IBGE 2012). According to the Köppen climate classification, the climate is of the Cfa type, humid subtropical, with hot summers and no defined dry season (Alvarez *et al*. 2014). Annual rainfall ranges from 989.3 to 2748.7 mm (Ribeiro *et al*. 2012). The predominant soil is classified as Rhodic Hapludox (Soil Survey Staff 2014). Voucher material was deposited in the herbarium of Pontifícia Universidade Católica do Rio Grande do Sul (MPUC) under number 22640.

Flower morphology

To describe the floral morphology of the *P. interior*, we recorded measurements of the diameter of the open corolla (limb), length of the floral tube and of the nectar chamber for 20 flowers (one flower/individual). To count the number of ovules, we dissected 30 gynoecia under a stereomicroscope, and to estimate the number of pollen per flower, we counted the pollen present in 30 anthers of pre-anthesis buds, from different individuals (one anther/bud/individual), using a Neubauer chamber (Moura *et al*. 2001) and an optical microscope. In addition, dehiscent anthers were crushed between slide and cover slip with Sudan III (Sass 1951) to verify the presence of lipid compounds in the locule fluid. The analyses and photomicrographs of the histological material were performed under a bright-field microscope with an Olympus CX31 microscope and a digital camera coupled to it.

Flower biology

To verify how many flowers were available to pollinators, we counted the number of open flowers in 20 plants, for two consecutive days, from 7:30 a.m. to 6:30 p.m., in October 2019. On one of these days, we also observed the opening and senescence pattern of the flowers/individuals. To verify whether floral longevity in unvisited and visited flowers differed, we measured the period that 10 bagged flowers and 10 unbagged flowers remained open to visitors. We observed the opening of the corolla and dehiscence of the anthers in 20 bagged flowers, recording the opening stage of the corolla and anthers every 30 minutes. To test the stigma receptivity, we used the peroxidase activity method (Dafni 1992) in six bagged flowers/hour (between 8:00 a.m. and 4:00 p.m.) during three anthesis days, in two repetitions (n = 108 flowers analyzed/anthesis day). We verified the presence of pollen self-deposition in the stigma in eight bagged flowers/hour (between 8:00 a.m. and 4:00 p.m.), during three anthesis days, in two repetitions (n = 144 flowers analyzed/anthesis day). Pollen viability was estimated by the pollen grain stain ability, measured by the colorimetric test using acetic carmine (Radford *et al*. 1974), with red-colored grains being considered viable. For this, we used five flowers in the early anther dehiscence stage, and one slide was prepared

for each flower anther (n = 25 anthers). We counted 300 grains per slide and the percentage of pollen that exhibited the appropriate color reaction was determined.

To describe nectar dynamics of *Petunia interior*, we assessed secretion and concentration hourly, from 8:00 a.m. to 4:00 p.m., using a Hamilton microsyringe (10 μL) and a portable refractometer, model RT-30ATC. We collected the accumulated nectar in eight flowers/hour (bagged in buds) during three anthesis days, in two repetitions, (n = 144 flowers analyzed/anthesis day). Standing crop nectar was collected in 20 flowers/hour, during two days (n = 360 flowers in total). To check the composition of the nectar, we collected this resource from 540 bagged flowers, and stored it in three Eppendorf tubes (nectar from 180 flowers in each tube). Subsequently, they were frozen at -80 ºC until analysis. The methodology proposed by Macrae & Zand-Moghaddam (1978) and Quemener (1988) was adapted for the analysis, with triplicates of samples (~ 15.0 mg) subjected to extraction with 115 μL of CH_3OH/H_2O (4:6, v/v) in water bath at 80 ºC for 30 minutes, under agitation. The solution was centrifuged at 5,000 g for five minutes and the supernatant transferred to a microtube. This extraction procedure was repeated two more times with purified water, with the supernatant stored in the same microtube and the volume of each sample adjusted to 375 μL. Activated carbon (10 mg/mL) was added to the extract and centrifuged at 5,000 g again for five minutes. The supernatant was diluted in acetonitrile 1:1 (v/v) and used directly for chromatography. To determine nectar sugar composition, we used a High-performance liquid chromatography (HPLC). The detection was performed by an Evaporative light scattering detector (ELSD), the samples were kept at room temperature (~ 22 °C) and the column temperature was at 50°C. Separations were performed on a Gist NH_2 column (Shimadzu) with 150 mm x 4.6 mm i.d. and 5 μm particle size. The mobile phase consisted of acetonitrile: ultrapure water (85:15), with a flow of 1 mL min^{-1}, with separation achieved in less than 12 minutes. We prepared standard curves with glucose, fructose and sucrose standards (Merck), at concentrations of 100, 250, 500, 750, 1000, and 2000 μg mL^{-1}.

Reproductive biology

To verify the breeding mode of *P. interior* we performed pollination tests following Radford *et al*. (1974). For this, we established three treatments: spontaneous self-pollination (SSP) – bagged flowers were maintained closed; hand self-pollination (HSP) – bagged flowers were pollinated with pollen from the same flower; and hand cross-pollination (HCP) – stigmas pollinated with different pollen donors, at least 10 m distant. We also marked flowers exposed to floral visitors to assess reproductive success under natural conditions (Control Pollination – CP). Thirty flowers of 20 different plants were used for each treatment and the fruit set was determined (percentage of set fruits per treatment). We kept all flowers under protection until maturity, when the fruits were collected. For each treatment and control pollination, we recorded the following variables: the number of fruits formed, longitudinal and transverse circumference of the fruits, mass of the fruits and number of seeds per fruit.

To calculate the index of self-incompatibility (ISI), we used the formula proposed by Lloyd (1965):

$$ISI = 1 - \frac{no.\ of\ fruits\ formed\ by\ manual\ self\ -\ pollination}{no.\ of\ fruits\ formed\ by\ manual\ cross\ -\ pollination}$$

To classify the values obtained in ISI, we follow the methodology used by Raduski *et al*. (2012): xenogamous – ISI ≥ 0.8; partially xenogamous – 0.2 < ISI < 0.8; autogamous – ISI ≤ 0.2.

Flower visitors and pollinators

Flower visitors were collected throughout the flowering season, in October and November 2018, with entomological nets at different times of the day, on non-consecutive days. The specimens were mounted, identified and deposited in the entomological collection of Pontifícia Universidade Católica do Rio Grande do Sul (MPUC). After the visitors were identified and researchers received training for recognizing them in the field, the frequency of visits was recorded. The records were made for flowers present in a visual field of one square meter, for 30 minutes per hour, from 8:00 a.m. to 5:00 p.m., on non-consecutive days in the months of October and November 2019, totaling 30 hours of direct observation. We recorded the number of flowers visited by species of visitor and the type of floral resource that each species collected. Flower visitors who visited the flowers, carried pollen and touched the stigma were considered pollinators (Alves-dos-Santos *et al*. 2016).

Data analysis

Means and standard error (±) of morphological measurements, mean number of ovules and pollen grains per flower, percentage of viable pollen grains, receptive stigmas and pollen autodeposition were calculated using Microsoft Office Excel 2007 (Microsoft). The other analyses were conducted using the statistical computing software R (R Development Core Team 2019). All data were submitted to analysis of variance (ANOVA), normality test (Shapiro–Wilk) and homoscedasticity (Samiuddin) tests, at a significance of 5 %. The number of open flowers and the number of floral visitors were analyzed based on time of day (7 a.m. to 6 p.m.) through the Skott-Knott test, using the ExpDes.pt package (Ferreira *et al*. 2018). Floral longevity between bagged and unbagged flowers was submitted to Student's t-test. The relationships between time of day (8 a.m. to 4 p.m.) and the volume and concentration of accumulated nectar were calculated using linear regression models. The contrast in the concentration of sugars (glucose, fructose and sucrose) was determined by Tukey's test. Pollination treatments did not exhibit normal residuals and were compared by Kruskal–Wallis one-way analysis of variance, using the Agricolae package (Mendiburu 2019).

Results

Flower morphology

Petunia interior has hermaphroditic and heterochlamydeous flowers. The calyx is pentamer, polysepalous and greenish in color (Fig. 1A). The corolla is infundibuliform, pentamer, gamopetalous, zygomorphic and purple (Fig. 1A, B), with diameter of the limbs between 18 and 25 mm (22.0 ± 2.13; n = 20) and length of the floral tube between 12 and 19 mm (15.9 ± 1.59; n = 20). The androecium is isostemonous, inserted and epipetalous, formed by five free and heterodynamic stamens (two large, two medium and one small) with dorsifixed and longitudinal anthers containing violet pollen (Fig. 1C). The connation of the

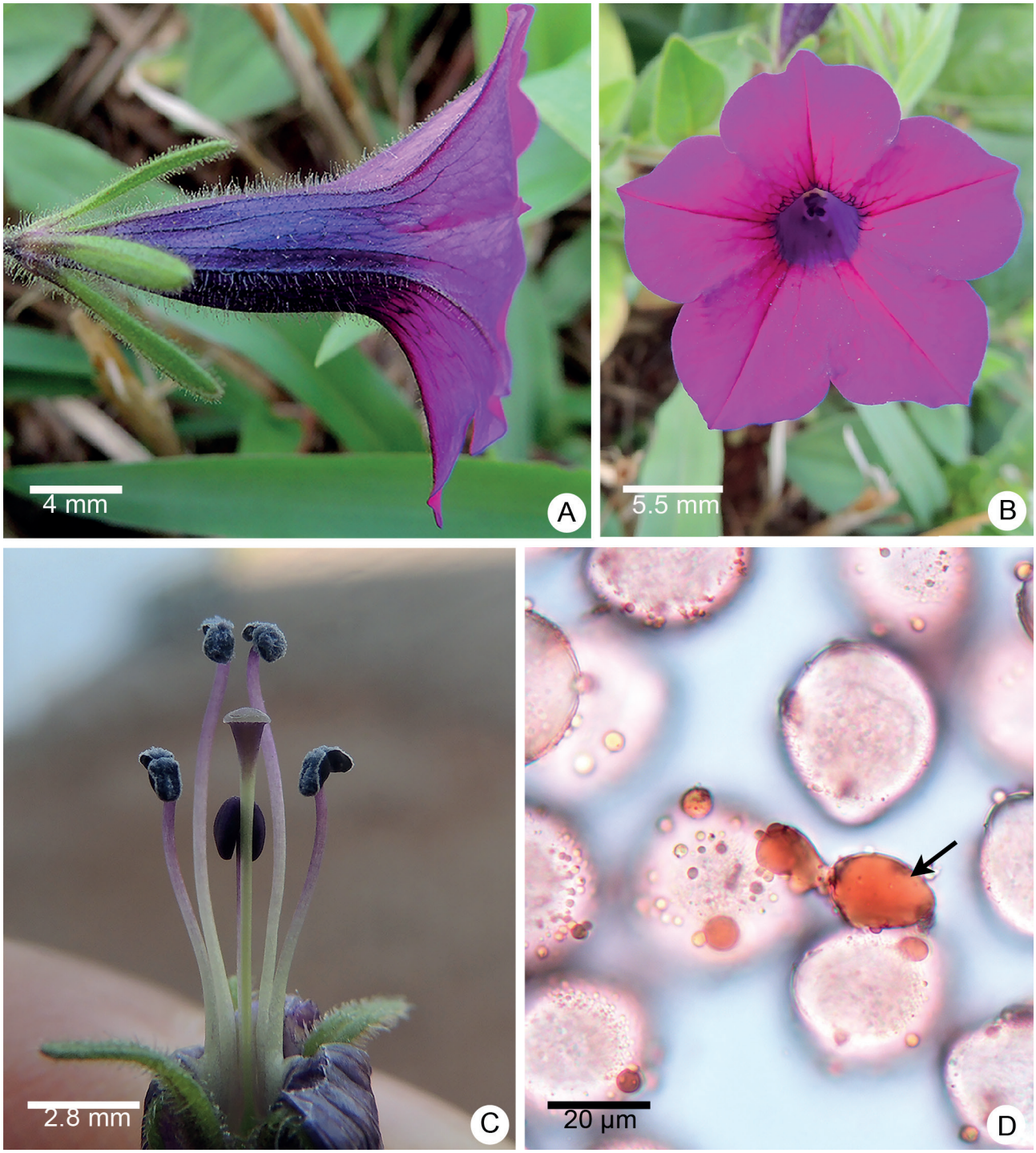

Figure 1. Flower morphology of *Petunia interior*. **A)** Flower from the side. **B)** Frontal view of the flower. **C)** Androecium and gynoecium. **D)** Positive reaction for lipids in the anther locular fluid (black arrow).

filaments in the corolla delimits a nectar chamber with an average length of 4.2 ± 0.51 mm (n = 20). The gynoecium has a superior ovary with bilobed nectary at the base and terminal style. The stigma is truncated and located between the anthers of the medium and large stamens (Fig. 1C). The average number of ovules per ovary was 156.7 ± 267 (n = 30). The average number of pollen grains per anther was 15.906 ± 4.592, and per flower 79.533 ± 2.22964 (n = 30). External to the pollen grains, the presence of lipid compounds (Fig. 1D), which are part of the anther's locular fluid, was verified.

Flower biology

The *P. interior* flowers began to open around 8:00 a.m. (Fig. 2), and open flowers registered at 7:00 a.m. were those from the previous day. Complete expansion of the corolla occurred approximately one hour after the beginning of anthesis. Average flower longevity was significantly higher ($p < 0.0001$) for bagged flowers (61.7 ± 13.3 hours) compared to unbagged flowers (17.3 ± 9.44 hours). The opening and senescence of flowers per individual were non-synchronized throughout the day. At the population level, there was a gradual and significant increase in the number of flowers opened until mid-day, with a maximum average of 16.8 ± 1.44 flowers per individual opened between 12:00 and 12:30 (Fig. 2). After the 1:30 p.m. period, there was a significant decrease in the number of open flowers (Fig. 2). The anther dehiscence began around 30 minutes after the total opening of the flower. First, they opened the anthers of the large stamens, then the anthers of the medium stamens and, finally, the anthers of the small stamens. The opening of all anthers occurred in approximately one hour and thirty minutes. Stigmas were receptive from the bud phase. On the first day of anthesis, 100 % (n = 108) of the analyzed flowers had receptive stigmas. The receptivity decreased in flowers on the second day (88 %, n = 108) and third day (26 %, n = 108) of anthesis. Self-deposition of pollen on flower stigma was recorded only on the second (10 %, n = 144) and third (12 %, n = 144) anthesis day after the flower opened. Pollen viability was 95.8 % and all anthers (n = 25) exhibited viable pollen grains.

Linear regressions revealed a significant relationship between times of day and volume (Fig. 3A), and the concentration of accumulated nectar (Fig. 3B). The linear regression showed that nectar volume increased the day (dotted with small grid in Fig. 3A). The flowers had a higher mean volume (2.4 ± 0.7 µL) on the second day of anthesis (see dashed lines in Fig. 3A) when compared to the first (1.5 ± 0.5 µL) and third day (1.2 ± 0.6 µL). Nectar concentration increased between the first (16.4 ± 7.3 %), second (21.1 ± 9.0 %) and third (23.6 ± 7.7 %) anthesis day. Despite this, nectar concentration remained practically constant on the first (dashed lines) and third days (dashed-dot lines) (Fig. 3B), while linear regression showed that nectar concentration decreased over the second day of anthesis (long dash line) (Fig 3B). Linear regressions were not significant for volume (0.02 ± 0.01 µL) and concentration (14.4 ± 1.2 %) of standing crop nectar. Three main sugars were identified in the analysis of the composition of soluble solids in the nectar. The concentration of sugars was calculated by external standardization, using their respective standard curves: glucose ($y = 552.84x + 11214.6$, $R^2 = 0.99$); fructose ($y = 2575.10x - 123219$, $R^2 = 0.99$) and sucrose ($y = 2649.41x - 118561$, $R^2 = 0.99$). Among the soluble solids found in this resource, 37.3 % were composed of glucose, 23.3 % of fructose and 17 % of sucrose, while the remaining (22.4 %) were composed of other poorly concentrated oligosaccharides not labeled here. Considering only the three main sugars, the proportion, in decreasing order, was: glucose (48 %), fructose (30 %) and sucrose (22 %).

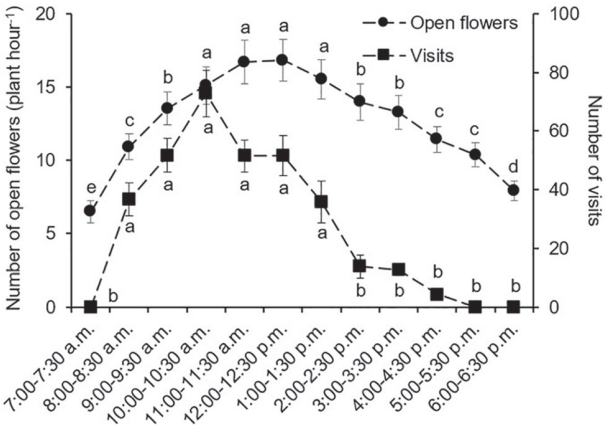

Figure 2. Number of open flowers (n = 20 plants) and floral visitors per hour during the study period. Dots correspond to mean values ± standard error. The means followed by the same letter are not significantly different (Scott-Knott clustering test, $P \leq 0.05$).

Reproductive Biology

Petunia interior presented a high percentage of fruit formed through hand cross-pollination (90 %) and control pollination (97 %). In contrast, this species exhibited a low percentage of fruit formed from hand self-pollination (10 %), and no fruit was formed by spontaneous self-pollination. The absolute difference (Δ %) between fruit formed in control pollination and the other tests (Tab. 1) shows that the plant reaches its maximum reproductive potential under natural conditions. Also, the registered ISI value was 0.9, which classifies the species as xenogamous. The average values of all variables registered for the fruits formed were significantly lower in those from self-pollinated flowers when compared to those resulting from hand cross-pollination and control pollination (Tab. 2). The values of mass and number of seeds of the fruits formed from hand cross-pollination and control pollination did not differ among themselves, while the values of diameter (transversal and longitudinal) were significantly higher for the hand cross-pollination treatment (Tab. 2).

Table 1. Breeding system of *Petunia interior:* fruit set after spontaneous self-pollination (SSP), hand self-pollination (HSP), hand cross-pollination (HCP), and controlled pollination (CP), during the study period, in Cerro Largo, Rio Grande do Sul, Brazil.

Treatment	N	Fruit set	Fruit set (%)
Spontaneous self-pollination	30	0	0
Hand self-pollination	30	3	10
Hand cross-pollination	30	27	90
Controlled pollination	30	29	97
Δ% (CP – SSP)		+97.0 %	
Δ% (CP – HSP)		+87.0 %	
Δ% (CP – HCP)		+7.0 %	

Flower visitors and pollinators

Bees of four species, belonging to three families, visited the *P. interior* flowers (Tab. 3). *Hexantheda missionica* was the most frequent species, with 62 % (n = 1162) of visits, followed by *Pseudogapostemon pruinosus* with 18 % (n = 342), *Callonychium petuniae* with 18 % (n = 335), and *Anthrenoides meloi* with 2 % (n = 40). The bees started the visits at the beginning of floral anthesis (around 8:00 am) (Fig. 2). Until the 1:30 pm period, no significant difference in number of visitors was observed, but there was a significant decrease after this period, together with a significant decrease in the number of open flowers per plant (Fig 2). Female bees took nectar and collected pollen from flowers. Males of three species were recorded taking nectar from the flowers (Tab. 3). Both male and female bees touched the anthers and stigma of the flower and were considered pollinators.

Discussion

Floral traits

Floral traits such as diurnal flower opening, short and wide corolla tubes, stamens with anthers inserted in the corolla tube, blue pollen, and purple corolla are presents in the melittophilous species of the genus *Petunia* (Wittmann *et al*. 1990; Ando *et al*. 2001; Castellani & Lopes 2002; Stehmann *et al*. 2009; Araújo *et al*. 2019). We found that *P. interior* shares these typical floral attributes of bee-pollinated species and was exclusively pollinated by bees. Regarding functional traits, although nectar volume corresponds to that proposed for bee-pollinated species of the genus (Gübitz *et al*. 2009), *P. interior*'s nectar sugar concentrations were lower than those observed in melittophilous species (Pamminger

Table 2. Average values of the variables recorded for the *Petunia interior* fruits in hand self-pollination (HSP), hand cross-pollination (HCP) and controlled pollination (CP), during the study period (Mean ± SD), in Cerro Largo, Rio Grande do Sul, Brazil.

Treatment	Fruit weight	Number of seeds	Fruit longitudinal diameter	Fruit transverse diameter
	mg	fruit^{-1}	mm	mm
HSP	3.1 ± 1.8 b	33.0 ± 1.8 b	3.9 ± 2.2 c	2.4 ± 1.4 c
HCP	17.2 ± 3.8 a	176 ± 1.4 a	6.0 ± 1.3 a	3.9 ± 0.9 a
CP	15.5 ± 3.0 a	175. ± 9.3 a	5.4 ± 1.1 b	3.5 ± 0.7 b
p-value	0.0044	0.0082	0.0005	< 0.0001

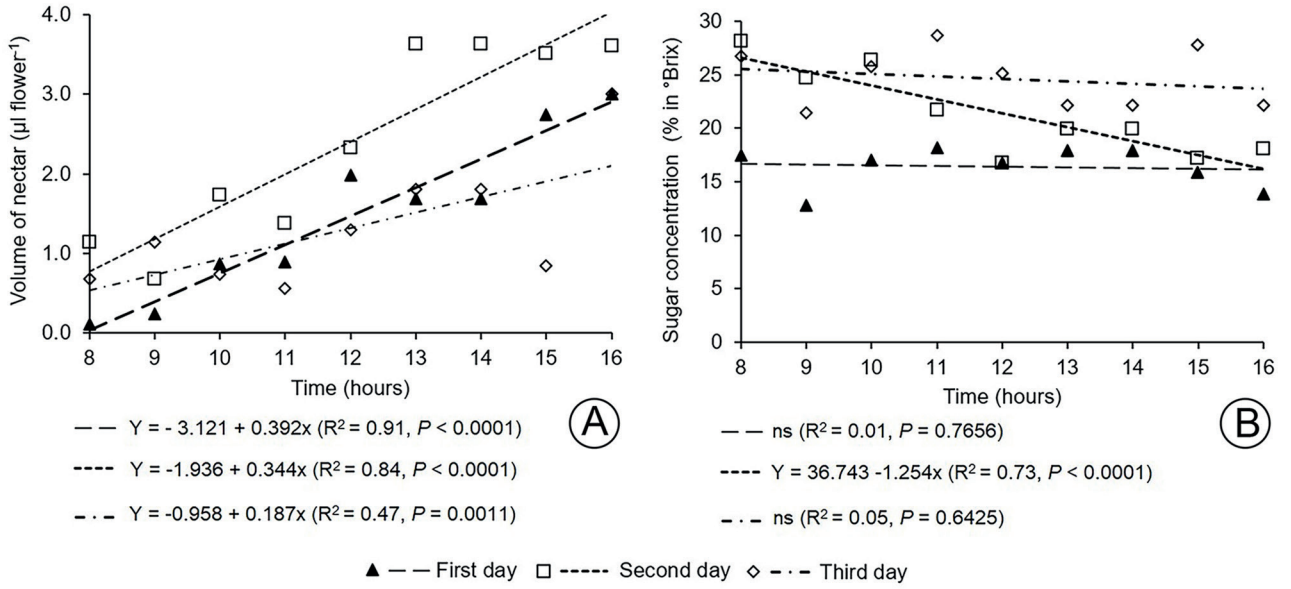

Figure 3. Linear regressions of the relationship between collection time and volume (**A**) and concentration of accumulated nectar (**B**). Sample size (N) for nectar volume (N = 144 flowers per day) and nectar sugar concentration (N = 120, 131 and 91 flowers on the first, second and third day, respectively). Statistically significant regressions ($P \leq 0.05$). Ns = not significant.

Table 3. Species of flower visitors to *Petunia interior* flowers in Cerro Largo, Rio Grande do Sul, Brazil, including sex, resource collected (RC); pollen (P); nectar (N), relative frequency of visits.

Bee family	Bee species	Sex	RC	% Visits
Andrenidae	*Anthrenoides meloi* Urban, 2005	♀/♂	P/N	2
	Callonychium petuniae Cure & Wittmann, 1990	♀/♂	P/N	18
Colletidae	*Hexantheda missionica* Oglobin, 1948	♀/♂	P/N	62
Halictidae	*Pseudogapostemon pruinosus* Moure & Sakagami, 1984	♀	P/N	18

et al. 2019). The bee optimal nectar concentration value ranges between 35-65 % (Roubik *et al*. 1995; Kim *et al*. 2011; Pamminger *et al*. 2019). Although some bees will collect nectar below these values under natural conditions (Roubik & Buchmann 1984), evidence indicates that they avoid concentrations below 20 % (Roubik & Buchmann 1984; Cnaani *et al*. 2006). However, in *P. interior* we found concentrations of 14.4 % in standing crop nectar and 16.4, 21.1 and 23.6 % in nectar accumulated from the first to the third day of anthesis, respectively. We emphasize here that although *P. interior* presents accumulated nectar with concentrations above 20 % on the second and third anthesis day, flowers of these days are rarely available in natural conditions, since floral longevity is reduced in these flowers. In addition, compared to known concentrations within the genus, these values differed from bee-pollinated species *P. integrifolia* (~ 37 %), but were like the values of the moth-pollinated species *P. axillaris* (~ 16 %) (Brandenburg *et al*. 2012). Thus, considering that the value of nectar concentration in *P. interior* flowers differs from the standard inferred for bee-pollinated petunias (See Stuurman *et al*. 2004; Gübitz *et al*. 2009), we suggest that further studies are needed to assess whether this decreased concentration is common in other bee-pollinated species of the genus. It should be noted that diluted nectar (~21 %) was also recorded for the bee-pollinated species *P. secreta*, but due to the long tubular flowers, this resource was inaccessible to bees (Rodrigues *et al*. 2018). Finally, even with concentration far from the standards, when comparing the accumulated volume (1.5 µL in first anthesis day) with the volume of standing crop nectar (0.02 µL), it becomes clear that this resource was intensely collected by bees.

Contrary to the values recorded for total concentration, the composition and proportion of the constituent sugars of *P. interior* nectar (sucrose = 22 %; glucose + fructose = 78 %) correspond to that known for bee-pollinated petunias, which present a proportion of sucrose lower than the proportion of glucose + fructose (Gübitz *et al*. 2009; Brandenburg *et al*. 2012). In *P. integrifolia,* Brandenburg *et al*. (2012) recorded a sucrose proportion of 35 %, and glucose + fructose of 55 %. These records are very interesting because they show that the variation in proportion of the types of sugars in the nectar of these two bee-pollinated species of *Petunia* does not interfere with the attraction of specific pollinators, since the oligolectic bees *Hexantheda missionica* and *Callonichium petuniae* were also registered in *P. integrifolia*. (Wittmann *et al*. 1990; Castellani & Lopes 2002).

Reproductive strategies

The self-incompatibility index of 0.9 indicates that *P. interior* is a xenogamous species, corroborating what has been known and suggested for the species of the genus that are purple, bee-pollinated and have blue pollen (Lee *et al*. 1994; Zhi-Hua *et al.* 2008; Castellani & Lopes 2002; Stehmann *et al*. 2009; Araújo *et al*. 2019).

In hermaphroditic flowers, the proximity of reproductive organs (absence of herkogamy) and the overlapping of the male and female phases (absence of dichogamy or incomplete dichogamy) commonly result in sexual interference and, consequently, in self-pollination (Lloyd & Webb 1986). However, despite the absence of spatial and temporal departure between reproductive organs in *P. interior* flowers, pollen self-deposition in the stigma was rarely recorded. Pollen self-deposition on the stigma of *P. interior* was possibly avoided due to the presence of lipid compounds in the locular fluid of the anthers. In the genus *Petunia*, these lipid compounds have been identified as pollenkitt, a substance that plays a role in pollen adhesion and transport, as it keeps the pollen grains together and attached to the anthers until a visitor removes a greater number of them (Pacini & Hesse 2005; Lin *et al*. 2013).

Petunia interior produces a considerable number of flowers that open daily and maintains its flowers open for a long period of the day, two aggregate traits that extend the likelihood to be visited by pollinators, which may ensure its reproductive success. Floral longevity has important consequences for pollination success, since it can ensure sufficient pollen receipt, increase donor diversity and promote pollen export (Primack 1985; Marshall *et al.* 2010; Fung & Thomson 2017). In addition, the average longevity recorded in *P. interior* flowers available to pollinators was about 3.6 times lower than the longevity of flowers that did not receive visits, and in this case, shortened longevities in visited flowers (which may have been pollinated) can promote visitation to unpollinated flowers (Fung & Thomson 2017). On the other hand, the magnitude of flower display is closely related to flower longevity (Harder & Johnson 2005), and plants with more flowers are more attractive to pollinators than plants with fewer flowers (Mitchell *et al*. 2004). However, in some cases, as the flower display increases, pollinators can visit more flowers from the same plant and favor geitonogamous self-pollination (Mitchell *et al*. 2004; Karron & Mitchell 2012). Nonetheless, in *P. interior* the asynchronous opening of the flowers (in an individual plant) and gradual dehiscence of

the anthers possibly reduces the simultaneous supply of pollen between the flowers, which can decrease the chances of geitonogamy (Barret 2003).

Because flower opening is not synchronized, either within or among individuals, during the day, *P. interior* flowers provide continuous supply of pollen and nectar. In addition, the gradual dehiscence of the anthers of individual flowers, as occurs in *P. interior*, is recognized as a trait responsible for limiting the amount of pollen that can be removed during a pollinator visit (Lloyd & Yates 1982). Therefore, floral visitors are induced to frequent a high number of flowers to obtain the necessary amount of this resource (Siriani-Oliviera *et al*. 2018) which, consequently, must intensify cross-pollination (Lloyd & Yates 1982). Furthermore, the continuous secretion of nectar during the three days of anthesis, and the availability of low volumes of this resource maintain the attractiveness of the flower and induces bees to visit several flowers (Willmer & Stone 2004), which should further favor the cross-pollination of the species. It should be noted that this self-incompatible plant has shown high reproductive success in natural conditions, an aspect that reinforces the idea that its flower traits work as strategies to promote high attractiveness to pollinators and maximize reproduction.

Reproductive success and interaction with bees

Petunia interior depends on bees for reproduction, and our findings have shown that the high reproductive success in natural conditions (97%) may be a result of the way the species regulates its interaction with bees, as well as a result of the efficiency of its pollinators. This species was visited by four species of bees and, among these, the oligolectic bees *Callonychium petuniae* and *Hexantheda missionica* were by far the most frequent pollinators.

In *P. interior*, the bees started foraging activities right when the first flowers opened, and the visitation rate followed the abundance of open flowers throughout the day. The timing of floral resource availability has obvious consequences for the foraging behavior of the floral visiting bees since they can exhibit strong synchronization between the visitation period, the anthesis events, and the supply of flower resources (Linsley 1958; Wcislo & Cane 1996; Schlindwein & Wittmann 1997). Adjustment of pollen foraging to new flowers was recorded in *Pseudogapostemon fluminensis*, the *Petunia mantiqueirensis* pollinator (Araújo *et al*. 2019), and in a few oligolectic Colletidae bees (Schlindwein & Wittmann 1997; Siriani-Oliveira *et al*. 2018; 2019). In *P. mantiqueirensis*, pollen is the primary floral resource and females of *P. fluminensis* avoid old pollen-empty flowers (Araújo *et al*. 2019). On the other hand, in *P. interior* flowers, bees feed on pollen and nectar, and even after the depletion of pollen, the flowers were visited by bees looking for nectar (our personal observation).

In *P. interior*, pollination by oligolectic bees may be another important aspect that contributes to its reproductive success. As the intensity of visits between the flowers, and the efficiency in pollen deposition increased with the presence of specialized pollinators (Schlindwein & Wittmann 1997; Siriani-Oliveira *et al*. 2018; 2019), cross-pollination tends to be highly effective for this species. In this regard, in addition to greater reproductive success, the fruits formed by control pollination showed no significant difference in mass and number of seeds when compared to the fruits formed by hand cross-pollination, which highlights the efficiency and importance of the bees for the reproductive success of *P. interior* at our study site.

Oligolectic bees restrict larval pollen diet to few plants of a given genus or family (Cane & Sipes 2006) and are highly dependent on the availability of the host plants to which they are specialized. In the region of this study, *P. interior* occurs naturally in areas with high predominance of agricultural practices, where a reduction of populations of this species is noticeable (our personal observation). So, as a consequence of a decrease in floral resources, the mutual relationship between *P. interior* and oligolectic bees is on the way to being threatened in this region, with negative effects for both.

Based on the results of the present study, we have concluded that *P. interior* is pollinated exclusively by bees. Regarding its floral traits, only the sugar concentration in nectar does not correspond to bee pollination syndrome. Finally, the high reproductive success of *P. interior* in natural conditions suggests that the flower attributes of this species, such as floral longevity, floral display, the presence of lipids in the anther, and continuous supply of resources, work together as reproductive strategies to promote high attractiveness to pollinators and high reproductive success in natural conditions. On the other hand, *P. interior* does not suffer from pollination deficit in the study area, which shows the high efficiency of its pollinators. However, the conservation of these mutualistic relationships depends on the conservation and sustainable use of land and biodiversity.

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