

Flower Structure and Reproductive Biology of *Bougainvillea stipitata* (Nyctaginaceae)

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Abstract: *Bougainvillea stipitata* displays inflorescences with three pendant greenish flowers. Flowers open at sunset and last five days. Flower perianth is constricted in the middle and forms a tube that ends in five lobes. Fragrance is emitted by the papillae located on the lobe margins of the perianth. The nectary is located at the base of the staminal tube. It secretes fructose-dominant nectar with amino acids, phenols and reduced acids. Nectar secretion is continuous during flower lifetime and the flowers do not recover the reward. Mainly moths visit flowers. Pollen load on stigmas indicates that most flowers received more than 50 pollen grains, which are deposited by pollinators within the first two days of the flower life. Although the low natural fruit set of this self-incompatible species may be due to intraplant pollen flow, pollinators play an essential role for *B. stipitata* fruit production.

Key words: Nyctaginaceae, *Bougainvillea stipitata*, nectary, nectar sugar composition, self-incompatibility, Sphingidae.

Introduction

The family Nyctaginaceae is distributed in all warmer parts of the world, mostly in the New World, with ca. 31 genera and 400 species (Bittrich and Kuhn, 1993^[3]). The family is commonly called “four o'clock” family because the flowers of *Mirabilis jalapa* open in the afternoon, and comprises several species with nocturnal flower opening and others with diurnal anthesis.

The genus *Bougainvillea* Juss. includes some of the most showy ornamentals of the tropics and subtropics, with a total of 18 species distributed in Central and South America (Mabberley, 1997^[17]). Most of the available information on this genus is related to taxonomic aspects (e.g., Heimerl, 1901^[12]; Standley, 1931^[26]; Toursarkissian, 1975^[29]; López, in press^[16]). Although information on reproductive biology of *Bougainvillea* species is scant, some data are available about breeding systems (Gillis, 1976^[11]; Gibbs, 1990^[10]), pollinators (Vogel, 1954^[31]; Gillis, 1976^[11]), nectar sugar composition (Percival, 1961^[19]; Forcone

et al., 1997^[7]), and the inability of some cultivars to mature fruits (Cooper, 1932^[4]). Most of these data came from two cultivated species (*B. glabra* Choisy and *B. spectabilis* Willd.). To our knowledge, the reproductive aspects of a wild species of *Bougainvillea* have not previously been reported.

Bougainvillea stipitata is a 3- to 6-m high tree with light green foliage. Native to Argentina, Bolivia and Brazil, this tree grows in arid and semiarid regions. In Argentina it is commonly called “tala falso” because of the similarities with native species of *Celtis* or “alfilerillo” whose thorns resemble pins. This species is easily recognizable from its congeners by its pendent inflorescences, green bracts, greenish and strongly constricted perianth and reflexed limbs (Heimerl, 1901^[12]; Standley, 1931^[26]; Toursarkissian, 1975^[29]; López, in press^[16]).

Since many of the floral characteristics, used by taxonomists in assessing relationships among taxa, represent adaptations to specific pollinators or pollinating mechanisms, it is useful to gain an understanding of the reproductive biology of the plants (e.g., Omduff, 1969^[18]; Stuessy, 1990^[27]; Anderson, 1995^[1]). This information, in turn, can provide clues to clarify the function of adaptations to different classes of pollinators, and to hypothesize about the mechanisms that are involved in the production of fruits. This study represents a contribution to the understanding of the floral and reproductive biology of *B. stipitata*.

Materials and Methods

Plants

We studied a natural population of 42 adult individuals of *B. stipitata* (ca. 4 m in height), growing near La Calera city, Colón Department, Córdoba Province, Argentina (S31°20'42", W64°20'01"). The site of the population presents a moderate grade of disturbance, mainly due to cattle grazing. Disturbance related to different human activities is a very common circumstance in the Chaco Serrano forests from Córdoba. A voucher specimen (López 130) is deposited in CORD, Museo Botánico de Córdoba. Fieldwork was carried out from November to April during two flowering seasons (1999–2001).

Inflorescence and flowers

To determine the developmental sequence of the inflorescence, ten branches from seven individuals were tagged. Flower duration was determined by tagging buds in 13 inflorescences (39 flowers) from two individuals and following their development until corolla wilting. During this period, flower size, colour changes, pollen availability and stigma receptivity (using hydrogen peroxide, Galen and Plowright, 1987^[8]) were noted. Plant material was fixed in 70% ethanol for the study of inflorescence structure, flower morphology and pollen viability.

To determine which part of the perianth emits fragrance, tube and limb portions of three flowers were placed in clean glass containers. After 5 h, smelling the contents of the glass containers assessed fragrance emission. In addition, fragrance emission was checked during daylight and night hours by smelling flowers directly on the plants. Fragrance emission was also examined by staining the flowers with neutral red (Kearns and Inouye, 1993^[13]).

Breeding system

To determine the degree of compatibility, four individuals were used as donor and recipient plants for hand pollination treatments. Branches with buds were labelled and bagged with green cotton mesh (0.80 mm of pore diameter) to prevent pollinator visits. Flowers were treated as follows: (1) 286 bagged flowers without any treatment to test for spontaneous autogamy, (2) 163 hand-pollinated flowers with pollen from their own fresh dehiscent anthers to test for self-compatibility, (3) 61 hand-pollinated flowers with pollen from flowers of other plants to test for xenogamy, (4) 186 flowers left open to pollinators to evaluate natural pollination. Buds were emasculated by removing the parts of the tepals that contain the anthers before using them in outcrossing experiments; extreme care was taken to avoid damage to stigmata. For hand-pollinations, pollen grains obtained from fresh dehiscent anthers were deposited onto the receptive surface of the stigma with the aid of a narrow (0.20 mm wide) flexible leaf of a grass (*Stipa* sp.). Treated flowers were checked for fruit maturation every week. The self-incompatibility rate (ISI) was calculated according to Dafni (1992^[5]). In addition, 20 hand-pollinated flowers (10 for autogamy and 10 for xenogamy) were collected and fixed in 70% ethanol to observe pollen tubes. The gynoecium was dissected and softened with NaOH (10% aqueous solution) for 2 h, washed twice with distilled water, stained with aniline blue, squashed under a cover slip, then observed under a fluorescence microscope (Zeiss Axiophot equipped with a filter UV H 365 with an excitation wavelength of 365 nm).

Nectary and nectar

To study the nectary, four flowers were dehydrated through an ethyl alcohol-xylol series, and embedded in histoplast. Both cross and longitudinal sections were cut at 12 µm intervals, mounted serially, and stained with Toluidine blue (Sakai, 1973^[24]). To evaluate the presence and number of stomata in the nectary, the fused base of the filaments was dissected and cleared with NaOH (10% aqueous solution), washed with ethyl alcohol:acetic acid (3:1), and stained with an aqueous IKI solution. Observations were accomplished with an optical mi-

croscope with a graduated scale, and photomicrographs were taken with a Zeiss Axiophot microscope using Kodak T-Max film, 100 ASA.

Nectar was withdrawn from the flower using capillary glass tubes without removing the flowers from the plant. Extreme care was taken to avoid damage to the nectaries and other flower structures. Two variables were immediately measured: volume (µl), using graduated micropipettes, and sugar concentration (% sucrose; mass/total mass), with a pocket refractometer (Atago, Japan). The amount of sugar produced was expressed in mg, after Kearns and Inouye (1993^[13]). The nectar was stored on Whatman #1 chromatography paper. Tests for amino acids, lipids, phenols and reducing acids were performed after Baker and Baker (1975^[2]). Sugar separation was accomplished by gas chromatography. Nectar was lyophilized and silylated according to Sweeley et al. (1963^[28]). The derivatives were then injected into a Konik KNK 3000-HRGS gas chromatograph equipped with a Spectra-Physics SP 4290 data integrator, a flame ionization detector, and an OV 101 3% column (2 m long) on Chromosorb G/AW-DMCS (mesh 100–120). Nitrogen was the carrier gas (30 ml/min) and following temperature programme was used: 208 °C for 1 min, 1 °C/min until 215 °C, 10 °C/min until 280 °C for 2 min. Carbohydrate standards (Sigma Chem., St. Louis) were prepared using the same method. Sucrose (r) and hexose (hr) ratios were calculated as follows: sucrose/fructose + glucose and glucose/fructose, respectively. Nectar samples were collected from five different flower stages, representative of the different days of the flowers life.

Nectar production throughout anthesis was determined using sets of nine flowers each from eight plants. Buds were tagged for identification and bagged using tulle bags to prevent pollinator visits. Data were taken once for each set, allowing nectar to accumulate until measurement. An untouched new set was used for the measurement series. Nectar production rate (NPR) per hour was calculated as follows: µl of nectar produced between measurements divided by the hours between them (µl/h). The presence of nectar was also checked and quantified in buds to reveal nectar production in this early developmental stage of the flower.

Flower visitors

Diurnal observations were done from January to March throughout the day, for a total of 240 h. In addition, nocturnal observations were made between 20:00 to 24:00 and 20:00 to 9:00, covering a total of 97 h (32 and 65, respectively). Insect specimens were photographed for identification.

Pollen load on the stigma

In order to evaluate pollination intensity throughout anthesis, 120 stigmata from six trees (20 of each of the flower stages) were dissected. Then stigmata were stained with aniline blue in lactophenol, and pollen grains were counted under an optical microscope. Pollination efficiency was calculated as follows: (mean pollen grains on a stigma/mean pollen grains produced per flower) × 100 (Richards, 1997^[22]). To obtain the number of pollen grains produced per flower, one anther per flower was dissected in a drop of lactic acid:glycerin (3:1) on a microscope slide, and all pollen grains were counted under a

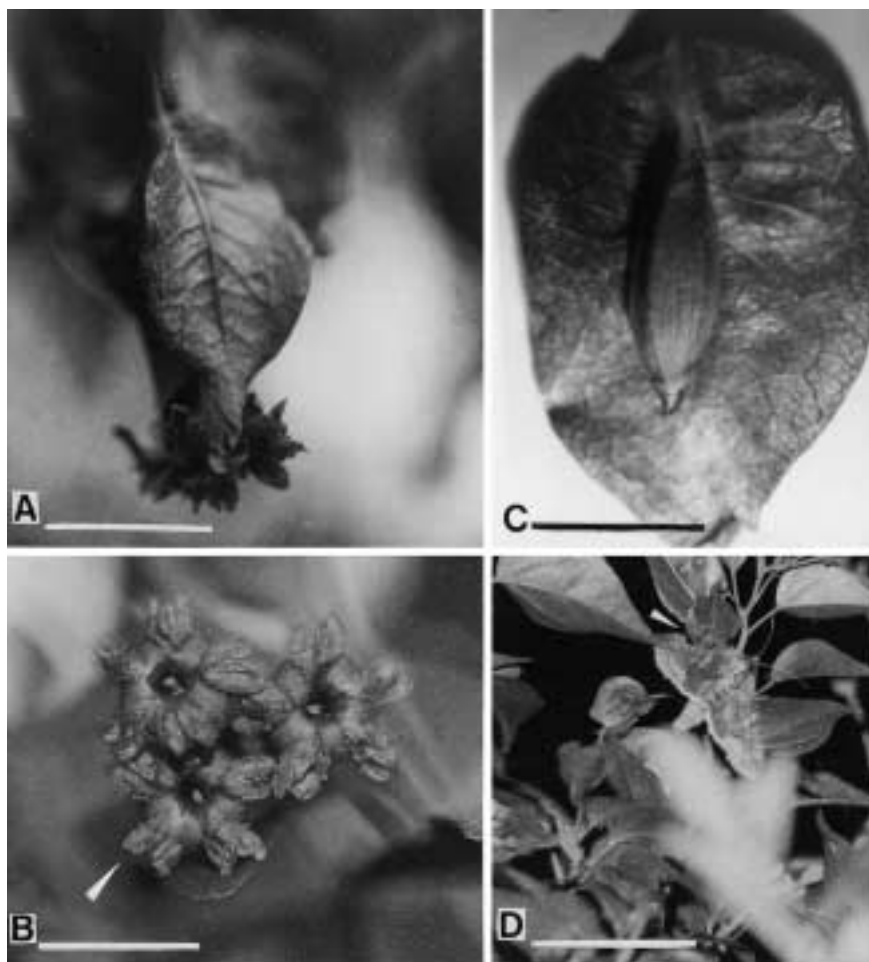


Fig. 1 *Bougainvillea stipitata*. (A) Inflorescence lateral view. (B) Inflorescence front view with one-day-old flowers; the arrow indicates the osmophores. (C) A mature fruit attached to the axillary bract. (D) A moth (fam. Sphingidae) visiting an inflorescence indicated by the arrow. Scale values = 1 cm for A and B, 0.50 cm for C and 3 cm for D.

stereo binocular microscope. Then this number was multiplied by eight (i.e., the number of anthers per flower). This procedure was repeated in three flowers of six different trees.

Data analysis

Statistical analyses were performed using methods described in Spiegel (1997^[25]). Means of original data plus or minus one standard deviation (SD) are given in the text and tables. Fruit set between treatments was compared using the chi-square test.

Results

Inflorescence and flowers

Bougainvillea stipitata showed asynchronous flowering, with some differences in timing among individuals. Thus, within one population it was common to observe plants in early bud, plants in full bloom, or even plants with fruits, all at the same time. Flowering starts in mid-November and ends in mid-April (i.e., at the beginning of autumn in the southern hemisphere).

The floral unit is represented by a three-flowered umbel. Each axillary shoot develops one to three inflorescences. The inflorescence has three pendant flowers, which are borne on the upper surface of a green, yellowish-green or reddish bract (Figs. 1A, 2A). The flower pedicel is attached to the central midrib of the bract (Fig. 2A). Within the inflorescence, flower opening is consecutive.

Flowers are salver-shaped, constricted in the middle, and radially symmetric (Figs. 1B, 2A). The perianth is green to yellowish-green, tubular, 1–2.5 mm in diameter at its widest, and 17–19 mm in length (Figs. 2A, B). The tube is 5-angled (Fig. 3B) and the limb terminates in five adaxially papillate lobes. Each lobe shows a central segment 1–1.5 mm wide × 2–3.5 mm long, with the papillae located along the margins (Fig. 1B). These central lobes are longer than the adjacent commissural lobes, which are 1 mm wide × 2 mm long (Fig. 1B). The androecium consists of eight stamens (Fig. 2B) with long, flattened filaments of different lengths. The longest filament is ca. 17 mm long and protrudes over the perianth, the shortest is ca. 12 mm long, and the rest measure 13 to 16 mm. Filaments are basely fused, forming a short staminal tube (Fig. 3A, B). The yellow anthers dehisce through lateral slits. The gynoecium is unilocular and bears one anacampylotropous ovule (Figs. 2B, 3A). The style is 3 mm long and emerges laterally from the upper part of the ovary. The stigma is ellip-

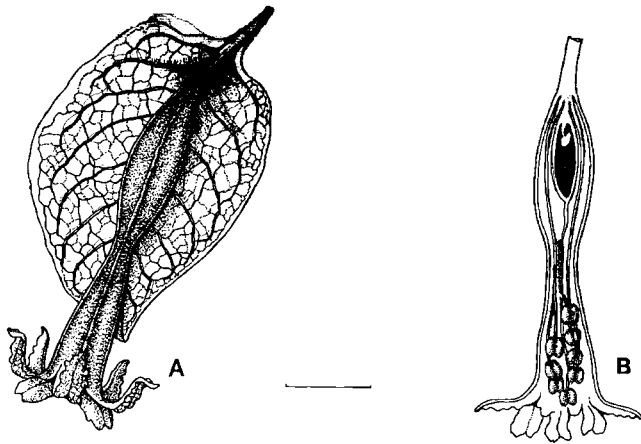


Fig. 2 *Bougainvillea stipitata*. (A) Lateral view of a one-day-old flower with its axillary bract. (B) Longitudinal section of a two-day-old flower showing the position of the stamens and the stigma. The scale bar represents 4 mm for A and B.

soid, 2 mm long, located on one side of the style. The receptive surface coincides with the constriction of the perianth and is located below the level of the anthers (Fig. 2B). One–two of three flowers of each inflorescence opens per day at sunset and lasts ca. five days. Flower lifetime was divided into five stages – essentially representing the days that a flower remains open –, as follows: (0) green-coloured buds ready to open, without nectar secretion; (1) flower at the beginning of anthesis with a green perianth, central and commissural lobes of the limb in a horizontal position, anthers dehiscent, tepals emitting fragrance, nectar present; (2) flowers as in stage 1, but central lobes reflexed; (3) yellowish-green flowers, position of the central and commissural lobes tending to be horizontal and vertical, respectively, nectar and fragrance present, anthers almost empty of pollen, stigma receptive; (4) flower colour and lobe position as in stage 3, nectar present but flowers without fragrance, anthers empty, stigmas remain receptive; (5) yellowish-green flower, central lobes starting to close, nectar present, stigma still receptive.

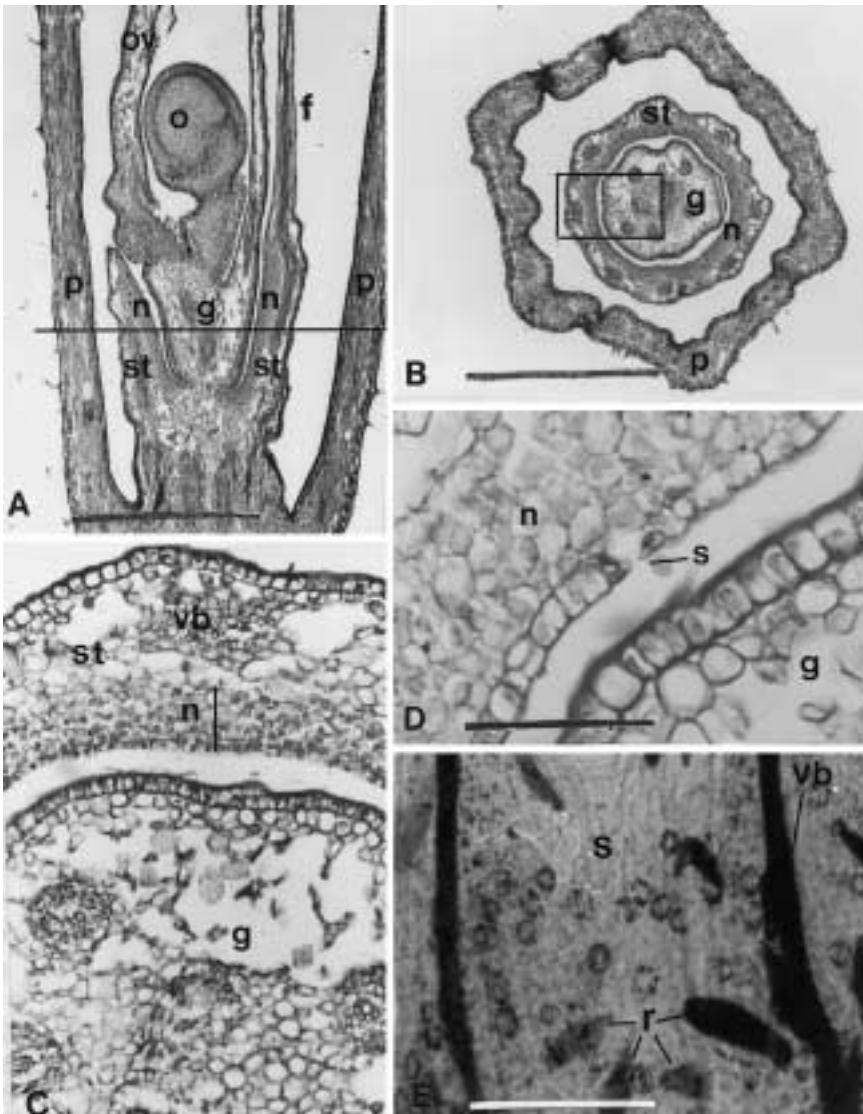


Fig. 3 Anatomical characteristics of the nectary of *Bougainvillea stipitata*. (A) Flower partial longitudinal section, the nectary tissue is localized in the adaxial surface of staminal tube. (B) Flower cross-section indicated in A showing the nectary. (C) Detail of the nectariferous tissue indicated in B. (D) Detail of a stoma from a cross-section of the adaxial surface of the nectary. (E) Detail of stained stomas of the nectary. Abbreviations: f = filament, g = gynoecium, n = nectariferous tissue, o = ovule, ov = ovary wall, p = perianth, r = raphides, st = staminal tube, s = stoma, vb = vascular bundles. The scale bars represent 1200 µm for A, 1330 µm for B, 220 µm for C, 81 µm for D and 290 µm for E.

Table 1 Nectar sugar composition of *Bougainvillea stipitata* throughout flower lifetime. Abbreviations: n = number of samples; r = sugar ratio; hr = hexose ratio

Flower stage	n	Sugar composition (%)			r	hr
		Sucrose	Glucose	Fructose		
1°	9	12.04 ± 7.84	4.74 ± 3.14	83.36 ± 11.12	0.136	0.057
2°	9	1.00 ± 1.41	31.10 ± 4.82	72.90 ± 3.66	0.010	0.427
3°	9	0.27 ± 0.46	24.11 ± 1.16	75.58 ± 1.02	0.003	0.319
4°	9	0	9.90 ± 1.48	90.11 ± 1.48	0	0.110
5°	9	0	26.94 ± 1.58	73.06 ± 1.57	0	0.369
Means of means	45	2.66 ± 5.26	19.36 ± 11.41	79.00 ± 7.53	0.030	0.256
Range		0–19.46	1.96–34.51	70.31–94.62		

Table 2 Breeding system of *Bougainvillea stipitata*

Treatments	Fruit set (# of fruits/ # of flowers)	# Fruiting plants/ # of plant used
Spontaneous self-pollination	0 (0/286)	0/4
Hand self-pollination	0 (0/163)	0/4
Hand cross-pollination	0.28 (17/61)	4/4
Natural pollination (Control)	0.35 (65/186)	4/4

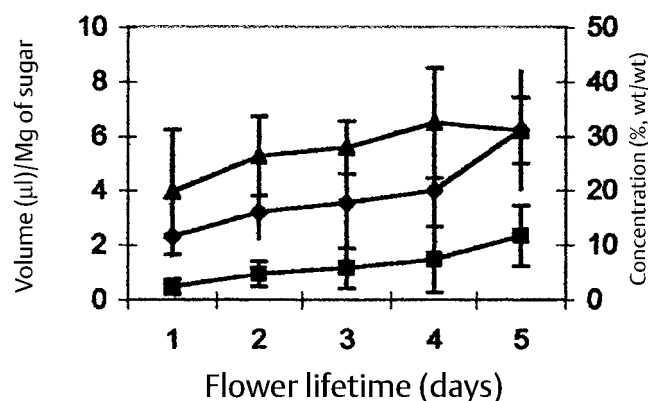
The test for odour and the positive neutral red staining of the lobes showed that the limb is responsible for fragrance emission. The papillae located on the lobe margins are functional. In addition, nocturnal fragrance emission was confirmed by direct inhalation of flowers of *in situ* plants. Fragrance emission was noticeable in the air 2–4 m from the plants.

Breeding system and fruit maturation

Bougainvillea stipitata matured fruits after hand cross pollinations. If unvisited or if self-pollinated, flowers did not set fruits. Thus, this species is classified as self-incompatible (ISI=0, Table 2). No significant differences were found between hand cross and natural pollination fruit set ($\chi^2=0.88$; $p>0.30$). Pollen tubes were observed growing into the stigma in the hand cross-pollination treatment, but they did not show fluorescence.

Among those inflorescences that receive compatible pollen, usually all three flowers produce fruits. If the flowers have been fertilized, the distal portion of the perianth – from the constriction zone upwards – shrivels and abscises in a few days. Fruit maturation takes ca. 30 days. The fruit is covered by the basal perianth tube, which forms an anthocarp, a unit that, together with the attached bract, helps wind dispersion (Fig. 1C). If pollination and/or fertilization did not occur, the entire inflorescence falls off two to five days after flowers open. Examination of some of these dropped inflorescences revealed the absence of fruits, further, pollen grains were not seen on stigmata.

Several flowers showed signs of predation caused by caterpillars which damaged and/or completely destroyed the gynoecium and the staminal tube. Caterpillars also damaged the em-

**Fig. 4** Nectar production of *Bougainvillea stipitata* throughout the flower lifetime. Symbols: diamonds = volume, triangles = concentration and squares = milligrams of sugar.

bryo and the perisperm in developing fruits. The percentage of predation (N = 20 individuals) was 3–20% for flowers and 3–40% for fruits.

Nectary and nectar

The nectary is green and located basally on the adaxial surface of the staminal tube; it forms a ring around the ovary (Figs. 3A–C). It is made up of six layers of cells (Figs. 3C,D). It is not supplied by a special vascular bundle. Raphides appear in the basal part of the nectary (Fig. 3E). Stomata are irregularly distributed in the upper half of the nectary surface (Fig. 3E). Stomata were always open. The nectary was persistent during fruit maturation.

Buds do not secrete nectar. Transparent nectar accumulates between the base of the stamens and the ovary. Test for nectar constituents revealed the presence of amino acids (0.03 mg/ml), phenols, and reducing acids. Lipids were not detected. Nectar sugar composition throughout the flower lifetime is presented in Table 1. Nectar was always fructose-dominant. When the flower opens, sucrose is present but quickly disappears as the flower ages. Mean nectar production per flower is shown in Fig. 4. Nectar secretion was constant throughout flower lifetime, with a mean NPRh of 0.032 mg/h. Nectar volume, concentration and sugar content are increased during anthesis (Fig. 4).

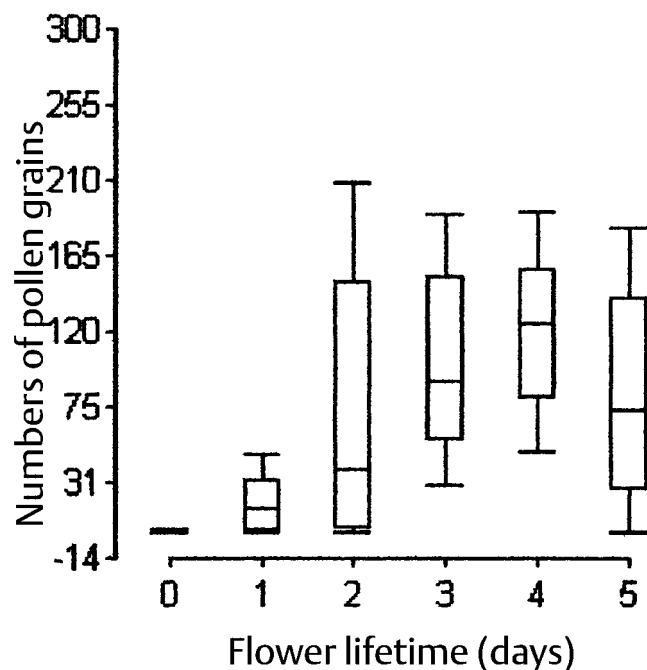


Fig. 5 Stigma pollen load found in *Bougainvillea stipitata* as the flower ages. Box plots represent medians and interquartile ranges.

Flower visitors

Only insects of the order Lepidoptera visited flowers. Butterflies were seen during daylight hours and moths during the night. A total of 18 individuals was recorded during more than 300 h of observation. Most visits (15) were nocturnal. Moths arrived at the trees after sunset (20:00 h; Fig. 1D, Fam. Sphingidae) and introduced their proboscis inside the perianth tube. The constriction of the perianth probably facilitates pollination, since it enforces contact between the proboscis and the stigma. Moths usually visited all three flowers of an inflorescence and several inflorescences per plant. Similar behaviour was observed for the butterfly (*Chioides* sp., Hesperidae) during daylight observations.

Pollen load

Because flower visitors were difficult to observe on the inflorescences, we collected and classified flowers according to the flower age in order to indirectly evaluate pollinator activity. Most flowers received pollen. Only 9% of stigmata did not present pollen grains. Recently open flowers showed fewer pollen grains than 2- or 3-day-old flowers (Fig. 5). In general, each flower received more than 50 pollen grains which are deposited by pollinators within the first two days of the flower life (Fig. 5). Mean pollen production per flower was 14102 ± 2886 pollen grains. Pollination efficiency was extremely low (less than 1%; range 0.2–9%).

Discussion

Flowers of *B. stipitata* showed traits (e.g., greenish tubular flowers, corolla constricted in the middle) that restrict visitors mainly because flower structure precludes all but pollinators with a thin proboscis longer than 15 mm. Fragrance emission

by flower tepals would be considered as an additional trait related to the attraction of a particular group of pollinators. Lepidoptera, specifically moths, can take advantage of the suite of characters presented by *B. stipitata* because of their morphological and physiological adaptations (Proctor et al., 1996^[21]). Other species of *Bougainvillea* present a different suite of traits to attract pollinators since they show coloured flowers and bracts and the absence of fragrance emission (e.g., *B. spectabilis* and *B. glabra*). The main advertisement in these species seems to be the showy colours of the inflorescences (i.e., mostly visual; Vogel, 1954^[31]). Considering flower and bract colour, fragrance emission seems to function as the principal advertisement in *B. stipitata* (i.e., mostly olfactory). Fragrance plays diverse roles in moth pollination, attracting moths from a distance, guiding floral approaches and feeding, among others (Levin et al., 2001^[15] and references therein). The fragrance apparently produces an effective moth attraction in *B. stipitata*, because pollinator visits occurred simultaneously with the period when fragrance was noticed in the air. In general, moth-pollinated flowers of unrelated species have similar morphologies and emit fragrance at night. Many authors have suggested that convergent evolution for moths' attraction in several angiosperm families may involve scent chemistry, as well as floral structure (e.g., Knudsen and Tollsten, 1993^[14]; Levin et al., 2001^[15] and references therein).

Nectar is the reward offered by *B. stipitata* to pollinators. It is produced by a nectary located at the base of the stamens that presents the usual structure and characteristics reported previously for other species of the genus (Rohweder and Huber, 1974^[23]; Zandonella, 1977^[32]; Vanvinckenroye et al., 1993^[30]). Nectar secretion possibly occurs via the stomas of the nectary through which nectar flows. *Bougainvillea stipitata* secreted hexose-dominant nectar, as do the other species of the genus investigated up to now (Percival, 1961^[19]; Forcone et al., 1997^[7]). Nectar sugar composition appears to be conservative within the genus and not related to the pollinator's preferences, because different pollinator guilds visit these species (Vogel, 1954^[31]; Gillis, 1976^[11]; H. López pers. obs.; data herein). In accordance with these findings in *Bougainvillea*, a relatively constant sugar composition was reported for three monophyletic groups of plants despite a large variety of pollinators visiting flowers of closely related species (Elisens and Freeman, 1988^[6]; Galetto et al., 1998^[9]; Perret et al., 2001^[20]). Upon this background, and specifically for *Bougainvillea*, nectary structure and nectar sugar composition appears to be more conservative than those traits related to flower structure and fragrance emission.

Bougainvillea stipitata secreted nectar at a relatively constant rate throughout the flower lifetime and the flowers do not recover this cost, indicating cost of nectar production may be low to the plant because of the large number of flowers produced during a flowering season. Because of the constant nectar secretion, probably all flowers provide a reward when moths arrive at an inflorescence. Data on stigma pollen load revealed that pollinators visited most of the flowers exposed to pollinators. The low natural fruit set of *B. stipitata* can be explained if moths, when searching for nectar, move most pollen within the same plant. Therefore, the chance for geitonogamous pollination is increased and the percentage of natural fruit set decreased. In other words, this species is not pollinator-limited, but limited by pollen quality because few flowers

received compatible pollen. Pollinators play an essential role for *B. stipitata* reproduction because this species did not produce fruits by selfing, but additional population attributes as density, distance to the nearest con-specific, among other factors, may also influence the production of seeds.

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