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Functional aspects of floral nectar secretion of *Ananas ananassoides*, an ornithophilous bromeliad from the Brazilian savanna

Juliana Marin Stahl[1], Massimo Nepi[2], Leonardo Galetto[3], Elza Guimarães[1,*] and Silvia Rodrigues Machado[1,*]

[1]*Department of Botany, Institute of Biosciences, UNESP–Univ Estadual Paulista, Campus de Botucatu, PO Box 510, SP, 18618-000, Brazil,* [2]*Department of Environmental Sciences, University of Siena, Via P. A. Mattioli 4, 53100 Siena, Italy and* [3]*Instituto Multidisciplinario de Biología Vegetal, Universidad Nacional de Córdoba, CONICET CC 495, 5000, Córdoba, Argentina*

** For correspondence. E-mail eguimaraes@ibb.unesp.br or smachado@ibb.unesp.br.*

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• *Background and Aims* Several members of Bromeliaceae show adaptations for hummingbird pollination in the Neotropics; however, the relationships between floral structure, nectar production, pollination and pollinators are poorly understood. The main goal of this study was to analyse the functional aspects of nectar secretion related to interaction with pollinators by evaluating floral biology, cellular and sub-cellular anatomy of the septal nectary and nectar composition of *Ananas ananassoides*, including an experimental approach to nectar dynamics.
• *Methods* Observations on floral anthesis and visitors were conducted in a population of *A. ananassoides* in the Brazilian savanna. Nectary samples were processed using standard methods for light and transmission electron microscopy. The main metabolites in nectary tissue were detected via histochemistry. Sugar composition was analysed by high-performance liquid chromatography (HPLC). The accumulated nectar was determined from bagged flowers ('unvisited'), and floral response to repeated nectar removal was evaluated in an experimental design simulating multiple visits by pollinators to the same flowers ('visited') over the course of anthesis.
• *Key Results* The hummingbirds *Hylocharis chrysura* and *Thalurania glaucopis* were the most frequent pollinators. The interlocular septal nectary, composed of three lenticular canals, extends from the ovary base to the style base. It consists of a secretory epithelium and nectary parenchyma rich in starch grains, which are hydrolysed during nectar secretion. The median volume of nectar in recently opened 'unvisited' flowers was 27·0 μL, with a mean (sucrose-dominated) sugar concentration of 30·5 %. Anthesis lasts approx. 11 h, and nectar secretion begins before sunrise. In 'visited' flowers (experimentally emptied every hour) the nectar total production per flower was significantly higher than in the 'unvisited' flowers (control) in terms of volume ($t = 4{\cdot}94$, $P = 0{\cdot}0001$) and mass of sugar ($t = 2{\cdot}95$, $P = 0{\cdot}007$), and the concentration was significantly lower ($t = 8{\cdot}04$, $P = 0{\cdot}0001$).
• *Conclusions* The data suggest that the total production of floral nectar in *A. ananassoides* is linked to the pollinators' activity and that the rapid renewal of nectar is related to the nectary morphological features.

Key words: *Ananas ananassoides*, Bromeliaceae, ornithophily, nectary structure, nectar secretion process, sugar composition.

INTRODUCTION

Species of Bromeliaceae are mainly ornithophilous and represent one of the most important energy sources for hummingbirds in Neotropical regions (Snow and Snow, 1986; Bernardello *et al.*, 1991; Sazima *et al.*, 1996; Buzato *et al.*, 2000; Krömer *et al.*, 2006). Nectar is the floral resource for hummingbirds and, in Bromeliaceae, it is produced by septal nectaries whose structure was described by Bernardello *et al.* (1991) and Sajo *et al.* (2004).

It has been suggested that the characteristics of Bromeliaceae nectar are predominantly determined by putative adaptations of nectar sugars to preferences of pollinators, rather than by phylogenetic relationships (Krömer *et al.*, 2006). Additionally, Stiles and Freeman (1993) verified that flowers associated with hummingbirds from distinct geographic regions shared a common sugar composition, indicating an adaptive convergence that reflects the taste preferences and/or the digestive physiology restrictions of hummingbirds.

As noted by McDade and Weeks (2004, p. 197), 'Despite the central role that nectar plays in mediating plant–pollinator interactions, for most plant species, we know little more than that nectar exists'. The authors follow by saying 'Clearly, we are far from having a complete understanding of the role of nectar in plant–pollinator interactions and of the evolution of nectar traits'.

Given these considerations, Bromeliaceae–hummingbird interactions represent a good model by which we may increase our knowledge of features of both the plant and the pollinator in the nectar-mediated interaction. Our focus was on the processes of nectar secretion including nectary characteristics, which could be informative in regard to the connection between nectar produced per flower and pollinator feeding behaviour. Additionally, we connected these results with a comparison of total nectar produced between 'visited' (i.e. nectar experimentally emptied) and 'unvisited' (control) flowers with the aim of identifying the effect of pollinator activity on plant physiological mechanisms related to both energy investment and saving.

MATERIALS AND METHODS

Study site and study organism

The study was conducted in a population of *Ananas ananassoides* (Baker) L.B.Sm. (Bromeliaceae) occurring at Reserva Particular Palmeira da Serra (22°48′50″S, 48°44′35″W), Pratânia municipality, São Paulo State, Brazil, in a cerrado *sensu stricto* phytophysiognomy (Brazilian savanna). The climate is characterized as Cwa (Köppen, 1948) and mesothermal, with rains in the summer and drought in the winter, and the median temperature of the hottest month is >22 °C (Cunha and Martins, 2009). Fieldwork was conducted during two consecutive flowering seasons of *A. ananassoides*. Flowering occurred at the beginning of the rainy season, spanning September to November 2008 and from September to October 2009.

This terrestrial bromeliad has leathery leaves and spiral phyllotaxis; a floral scape of approx. 1·3 m in length, with large, coloured bracts; a spike with many densely arranged flowers; sessile, tubular, trimerous flowers having lilac petals with stamens included and attached to the petal bases and a syncarpic gynoecium and inferior ovary with developed septal nectaries; and syncarpic, fleshy fruit (Wanderley and Martins, 2007). Voucher specimens were deposited in the Herbarium of the Department of Botany (BOTU) of the Institute of Biosciences, UNESP–Univ Estadual Paulista, Brazil, under numbers 24198 and 24193.

Plant–pollinator interactions

Floral morphology and events of anthesis were observed in ten plants, with emphasis on the opening time, colour of the floral elements, presence of nectar and floral longevity. Flowers were monitored to check for visitors at different times of day throughout the season totalling 60 h of observation, from dawn (0300 h) to evening (1700 h). Visitor behaviour was described based on field observations, analysis of photographs and video, recording the time, duration and frequency of visits, body regions that come into contact with the anther/stigma and the type of resources collected. Some visitors were captured for identification, and others, such as hummingbirds, were identified by photographs and video.

Structural and ultrastructural studies

For anatomical studies, samples were fixed in FAA 50 (formaldehyde, acetic acid, 50 % ethanol 1:1:18 v/v/v) (Johansen, 1940) for 24 h, followed by gradual dehydration in an ethanol series; the samples were then embedded in hydroxyethylmethacrylate (Leica Microsystems Inc., Heidelberger, Germany). Transverse and longitudinal sections (6–8 μm) were cut with a rotary microtome and stained with 0·05 % toluidine blue (pH 4·3) (O'Brien *et al.*, 1964). Histochemical analyses were performed according to the references in Table 1.

For scanning electron microscopy (SEM), fragments of ovary were isolated and fixed in 2·5 % glutaraldehyde (0·1 M phosphate buffer, pH 7·2), dehydrated in an ethanol series, dried to critical point and subsequently sputter-coated with approx. 10 nm gold as described by Robards (1978). The samples were examined in a scanning electron microscope, model Quanta 200 (Fei Company, Hillsboro, OR, USA), and all images were processed digitally.

For transmission electron microscopy (TEM), samples of nectaries obtained from the basal region of the ovary of functional flowers were fixed in glutaraldehyde (2·5 % with 0·1 M phosphate buffer, at pH 7·3) and left overnight at 4 °C. They were then post-fixed with 1 % osmium tetroxide (OsO_4) in the same buffer for 2 h at room temperature, dehydrated in a graded series of acetone solutions and embedded in Araldite resin. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and observed under a Philips TEM 100 microscope at 80 kV (Philips, Czech Republic).

Process of nectar secretion during anthesis and nectar sugar composition

The process of nectar secretion and the effect of nectar removal on the total energy and water content secreted during the lifetime of the flowers were investigated using all of the flowering individuals available in the population. Inflorescences with labelled pre-anthesis buds ($n = 50$ flowers, ten plants) were protected with bridal veil bags to prevent nectar depletion by visitors during the experiments, as recommended by Corbet (2003). The volume (μL) of nectar from open flowers was measured immediately after collection using graded syringes (Hamilton, USA). The sugar concentration was measured with a digital refractometer

TABLE 1. *Histochemistry of septal nectaries from functional flowers of* Ananas ananassoides

Staining procedure	Target compounds	References	Results*	Sites of reactivity
Sudan IV	Total lipids	Johansen (1940)	+	Ephithelial and parenchyma cells
NADI	Terpenes	David and Carde (1964)	+	Ephitelial cells and nectar channels
Schiff (PAS)	Neutral polysaccharides	Jensen (1962)	+	All nectary tissues
Lugol	Starch grains	Johansen (1940)	+	Parenchyma cells
Fehling's solution	Sugars	Sass (1951)	+	All nectary tissues
Rutenium red	Pectin/mucilage	Johansen (1940)	–	
Dragendorff	Alkaloids	Svendsen and Verpoorte (1983)	–	
Mercuric bromophenol blue	Proteins	Mazia *et al.* (1953)	–	
Ferric trichloride	Phenolic compounds	Johansen (1940)	–	Parenchyma cells
Sulfuric acid (5 %)	Crystals of calcium oxalate	Johansen (1940)	+	Parenchyma cells

* – negative; + positive.

(Callmex, Brazil) as per cent weight/weight sucrose (g sucrose per 100 g solution). These nectar data (volume and concentration) were used to estimate the total milligrams of sugar produced per flower using the exponential regression proposed by Galetto and Bernadello (2005).

To determine the pattern of nectar secretion throughout anthesis in the absence of nectarivores (i.e. accumulated nectar per flower), 11 groups of flowers were used, each one with three bagged flowers, referred to here as 'unvisited' flowers ($n = 33$ flowers, seven plants). Every hour from 0600 h to 1600 h, the nectar accumulated in each flower was withdrawn, and the volume and concentration were measured each time in a group of three flowers, which were then discarded, so that each of the 33 flowers was evaluated only once.

To evaluate the floral response to repeated nectar removals, one group of 17 flowers was used in an experimental design simulating multiple visits by pollinators (11 visits) to the same flowers over the course of anthesis (i.e. nectar experimentally emptied). This group was referred to here as 'visited' flowers ($n = 17$ flowers, three plants). In each flower of the 'visited' group the accumulated nectar was also withdrawn every hour from 0600 h to 1600 h, but in this treatment the nectar of each flower was drained 11 times. We summed the partial amounts for each flower (volume and milligrams of sugar obtained each hour) and averaged them to calculate the mean total production per flower during anthesis. Nectar measurements at 1700 h, in both treatments, 'visited' and 'unvisited', were not possible because the corolla began to wilt, preventing the entry of the syringe into the tube. We compared nectar production of 'unvisited' (control) with 'visited' (nectar experimentally emptied) flowers using volume, concentration and solute mass obtained from the following sets of data: for 'unvisited' flowers we used the values obtained from five groups of three flowers drained during the period comprised between 0900 h and 1300 h ($n = 15$ flowers, three plants), during which time nectar volume reached the maximum and remained almost constant; for 'visited' flowers we used values of total nectar produced per flower during the whole of anthesis ($n = 17$ flowers, three plants). The differences were evaluated by a *t*-test. The data of volume and mass of sugar showed different standard deviations, so the Welch correction was applied to perform the test.

For sugar composition analysis, samples of nectar (2 μL) were collected from open flowers of three plants at approx. 1000 h during October 2008. These samples were stored at −20 °C as dried spots on Whatman No. 1 filter paper as described by Galetto and Bernardello (2005). After the samples were thawed to ambient temperature, nectar was recovered from the filter paper by static elution with 100 μL of distilled water for 3–4 min, followed by centrifugation for 5 min at 11 000 *g*. The supernatant was analysed by isocratic high-performance liquid chromatography (HPLC) with the LC1 Waters system. A 20 μL aliquot of the sample and standard solution was injected. Water (MilliQ, pH 7) with a flow rate of 0·5 mL min^{-1} was used as the mobile phase. Sugars were separated in a Waters Sugar-Pack I column (6·5–300 mm), maintained at 90 °C, and were identified by a refractive index detector (Waters 2410).

RESULTS

Plant–pollinator interactions

Inflorescences are indeterminate, exhibiting 1–10 flowers in anthesis per day. Flower opening began at approx. 0300 h and was completed at 0600 h, when the corolla lobes were completely recurved and the nectar accumulated inside the corolla tube was available to floral visitors. At the end of each day, at approx. 1700 h, the lilac petals presented a pink tonality, and the apices were curled into the corolla centre, preventing access by visitors to the tube. Anthesis lasted approx. 11 h.

Relatively small hummingbirds of two species, *Thalurania glaucopis* (Fig. 1A) and *Hylocharis chrysura* (Fig. 1B), performed several visit sequences between 0700 and 0900 h, acting as trapliners. They foraged on 5–10 flowers per inflorescence (i.e. the majority of open flowers of each inflorescence) and usually visited each flower once or twice during the same sequence. After 1000 h, visits to the inflorescences were less frequent but continued intermittently. Hummingbirds flew over the inflorescences for approx. 1 min. They then hovered in front of a flower and introduced their beak into the floral tube for 1–3 s. After that, they flew toward other flowers of the same plant or of neighbouring plants, or they landed on a nearby branch to perform systematic beak cleaning.

Individuals of two butterfly species, *Hamadryas februa* (Fig. 1C) and *Phoebis sennae* (Fig. 1D), also visited the flowers, landing outside the corolla and introducing their proboscides for 1–3 min into the floral tubes. Next, they

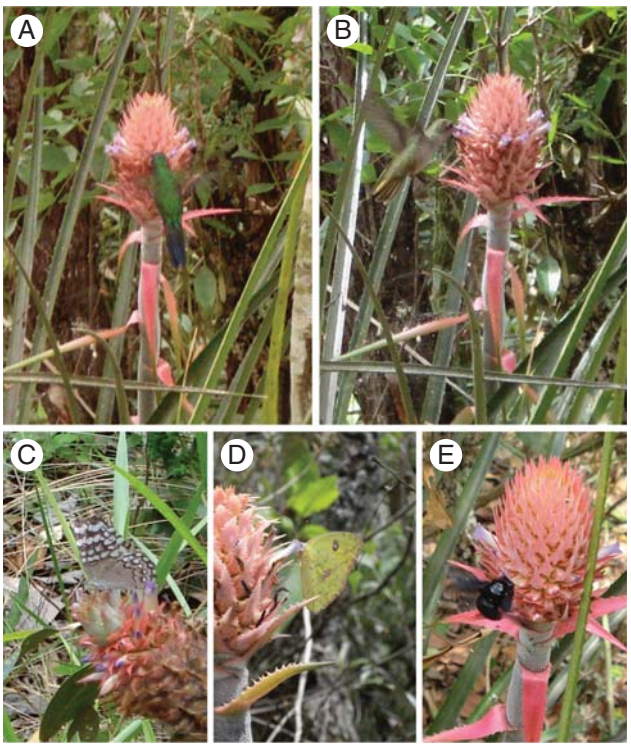

FIG. 1. Pollinators collecting nectar from functional flowers of *Ananas ananassoides*. (A) *Thalurania glaucopis* (Thochilidae). (B) *Hylocharis chrysura* (Thochilidae). (C) *Hamadryas februa* (Nymphalidae). (D) *Phoebis sennae* (Pieridae). (E) *Bombus morio* (Apidae).

TABLE 2. *Floral visitors of* Ananas ananassoides *in cerrado vegetation, Pratânia, SP, Brazil*

Species	Visit behaviour	Collected resource	Frequency*
Apidae			
Bombus morio	Legitimate	Nectar	Low
Plebeia droryana	Illegitimate	Pollen	High
Trigona spinipes	Illegitimate	Pollen	High
Unidentified species	Illegitimate	Pollen	High
Lepidoptera			
Hamadryas februa	Legitimate	Nectar	Low
Phoebis sennae	Legitimate	Nectar	Low
Trochilidae			
Hylocharis chrysura	Legitimate	Nectar	Medium
Thalurania glaucopis cf.	Legitimate	Nectar	Medium

* Frequency: high (about 30 visits d^{-1}), medium (1–5 visits d^{-1}), low (<1 visit d^{-1}).

would fly toward other flowers on the same plant or on neighbouring plants.

Large bees, *Bombus morio* (Fig. 1E), visited *A. ananassoides* flowers as legitimate pollinators, but they visited less frequently than hummingbirds and butterflies. *Bombus morio* individuals hovered in front of flowers and placed part of their heads into the floral tubes, coming into contact with the reproductive structures during apparent nectar collection. Small-bodied individuals of three bee species, *Trigona spinipes*, *Plebeia droryana* and one unidentified species, visited *A. ananassoides* flowers for pollen. These species seemed to act primarily as pollen thieves and may have caused self-pollination.

Flower visitors, feeding behaviours (legitimate or illegitimate), nature of the reward apparently collected and frequency of visits are summarized in Table 2. We assumed legitimate floral visitors to be those who, during resource collection, were able to perform cross-pollination through contact with the anthers, which were filled with pollen, and the receptive stigma, and that visited different individuals of *A. ananassoides* sequentially.

Nectary structure and ultrastructure

The septal nectary in *A. ananassoides* extends from the base of the ovary locules to the base of the style, where it opens to the base of the corolla tube. The nectar-secreting channels exhibit a convoluted and undulating outline (Fig. 2A) that is more developed at the basal region of the ovary (Fig. 2B). At or above the ovule attachment region, the channels present a linear outline (Fig. 2E) and have progressively less secretory tissue (Fig. 2F), ending in three apical pores through which the nectar flows.

In the secretory phase, the convolute region of the septal nectary (Fig. 2B) comprises two well-delimited regions in cross-section: an epithelium composed of 1–3 layers of juxtaposed, columnar cells that are disposed perpendicular to the septal nectary surface, and a differentiated nectary parenchyma composed of 3–6 layers of smaller, isodiametric cells (Fig. 2G). Cuticle was indistinguishable in this nectary region but it was visible on the non-secretory surface (Fig. 2F). The cells of the nectary parenchyma present a denser cytoplasm than the ground parenchyma cells (Fig. 2F). The septal nectaries lack an individual vascular supply, but vascular bundles (Fig. 2C) composed of phloem and primarily xylem elements occur near the nectary parenchyma tissue (Fig. 2B) without ramifying into it. The vascular parenchyma cells of both xylem and phloem contain dense cytoplasm, well-developed plastids with prominent starch grains, previously detected with the use of Lugol reagent, and numerous, small vacuoles (Fig. 2D). The presence of idioblasts with raphides (Fig. 2E, F, H) of calcium oxalate, confirmed with the use of sulfuric acid (5 %), is typical in the neighbouring nectary parenchyma.

Histochemical analyses of the nectary during the secretory stage were positive for lipophilic and hydrophilic substances (Table 1). Staining with Sudan IV revealed the presence of small oil droplets dispersed in the protoplast of epithelial and parenchyma secretory cells. Treatment with NADI reagent clearly showed the presence of terpenes, which were observed as densely stained droplets both inside the epithelial and nectary parenchyma cells and in the periplasmic space (Fig. 2I); bodies stained with NADI that are larger in size and spherical or ellipsoid in shape also occur on the surface of the epithelial cells and inside the nectary channels. All regions of the nectary showed strong positive reactions for polysaccharides because of the abundance of starch grains. Treatment with both Lugol's reagent and Dragendorff's reagent confirmed the presence of darkly stained starch grains in the epithelial and nectary parenchyma cells at anthesis, and it was possible to detect clearly a gradual reduction in the size and abundance of starch grains toward the epithelial cells (Fig. 2J). Samples of the nectary that were treated with Fehling's reagent exhibited a positive reaction, indicating the presence of reducing sugars. Phenolic compounds, mucilage and proteins were absent in the epithelial and parenchyma cells.

The epithelial cells of secreting nectaries exhibit thin radial and tangential walls, large ellipsoidal nuclei, abundant cytoplasm and small vacuoles (Fig. 3A). The surface of the outer tangential wall facing the channel is electron dense and presents deposits of osmiophilic material intermixed with fibrilar wall material (Fig. 3C, E) that are released by the disintegration of the cell wall during the channel development that occurs by schizogenesis (S. R. Machado *et al.*, unpubl. res.). Ultrastructural analysis confirmed that epithelial cells in this region do not have cuticle (Fig. 3C, E, F). The plasma membrane has an irregular outline (Fig. 3B, C) and gives rise to the periplasmic space, which is more developed in the apical pole of the epithelial cell (Fig. 3D, F). These spaces contain paramural bodies (Fig 3E) and large lipophilic drops (Fig. 3F). Vesicles (Fig. 3C) and portions of endoplasmic reticulum (Fig. 3E) close to the plasma membrane are visible.

The cytoplasm of the epithelial cells stains densely and contains large drops of lipophilic material (Fig. 3B), mitochondria (Fig. 3B, E, I), polyribosomes (Fig. 3B, E), endoplasmic reticulum (Fig. 3B, E), dictyosomes with adjacent secretory vesicles (Fig. 3E, G) and plastids (Fig. 3E, I). Some plastids are devoid of thylakoid membranes, and contain a homogeneous stroma and 1–2 ovoid starch grains (Fig. 3E); other plastids, mainly in the subjacent layer, are well developed and filled with prominent starch grains (Fig. 3I). The vacuoles are variable in size and are electron lucent (Fig. 3A, E).

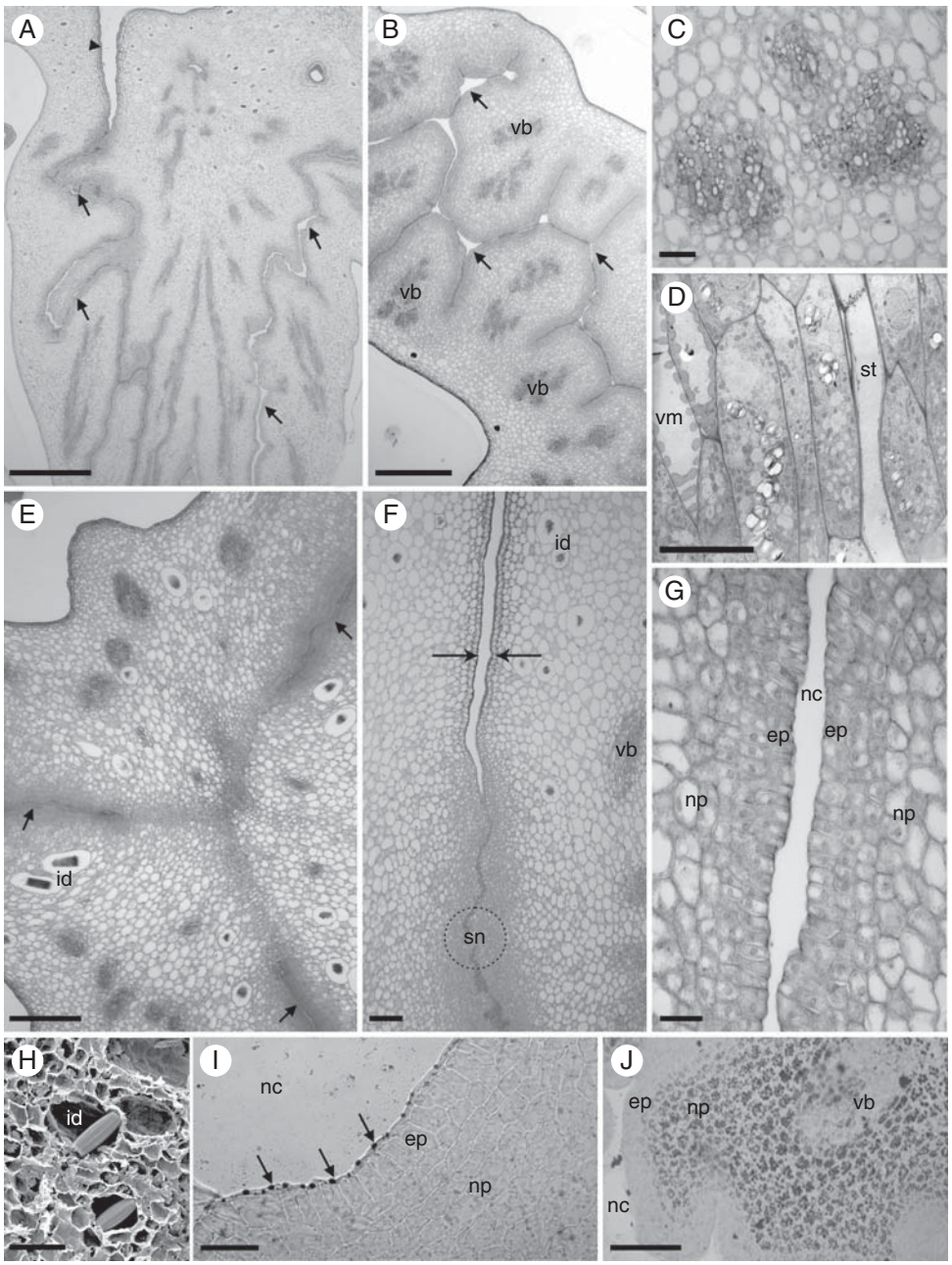

FIG. 2. Septal nectary structure of *Ananas ananassoides*. (A) Ovary longitudinal section showing septal nectary (arrows) and apical orifice (arrowhead). Scale bar = 500 μm. (B) Cross-section of the middle region of the ovary showing the nectar-secreting channel (arrows). vb, vascular bundle. Scale bar = 300 μm. (C) Detail of (B) showing vascular bundles. Scale bar = 150 μm. (D) TEM image of the vascular bundle showing vessel member (vm) and sieve tube member (st); note large amyloplasts in the parenchyma cells. Scale bar = 5 μm. (E) Cross-section of the ovary at the ovule attachment region showing the nectar-secreting channels (arrows) with a linear outline; note idioblasts with raphides (id). Scale bar = 200 μm. (F) Part of the ovary apical region showing a progressive lack of secretory tissue. Note the epidermis with cuticle (arrows), septal nectary (sn) and idioblasts (id) with raphides. Scale bar = 50 μm. (G) Part of the septal nectary in cross-section with multiseriate epithelium (ep), nectary parenchyma (np) and nectar-secreting channel (nc). Scale bar = 50 μm. (H) SEM image of the ovary showing idioblasts (id) with raphides. Scale bar = 20 μm. (I) Section of an NADI-stained nectary showing reaction products (arrows) between the protoplast and the cell wall of epithelial cells. Scale bar = 50 μm. (J) Distribution of starch grains in the septal nectary treated with Lugol's reagent. ep, epithelium; np, nectary parenchyma; vb, vascular bundle. Scale bar = 100 μm.

Plasmodesmata are very common in the anticlinal (Fig. 3G) and periclinal walls (Fig. 3H) of the epithelial and parenchyma cells. The nectary parenchyma cells (Fig. 3J) contain slightly lobed nuclei; the cytoplasm is less abundant than in epithelial cells and contains plentiful mitochondria and globe-shaped plastids that are filled with conspicuous starch grains; the vacuome is constituted by one central vacuole and numerous small ones in the periphery, and images suggesting the occurrence of fusion of vacuoles are observed in these cells.

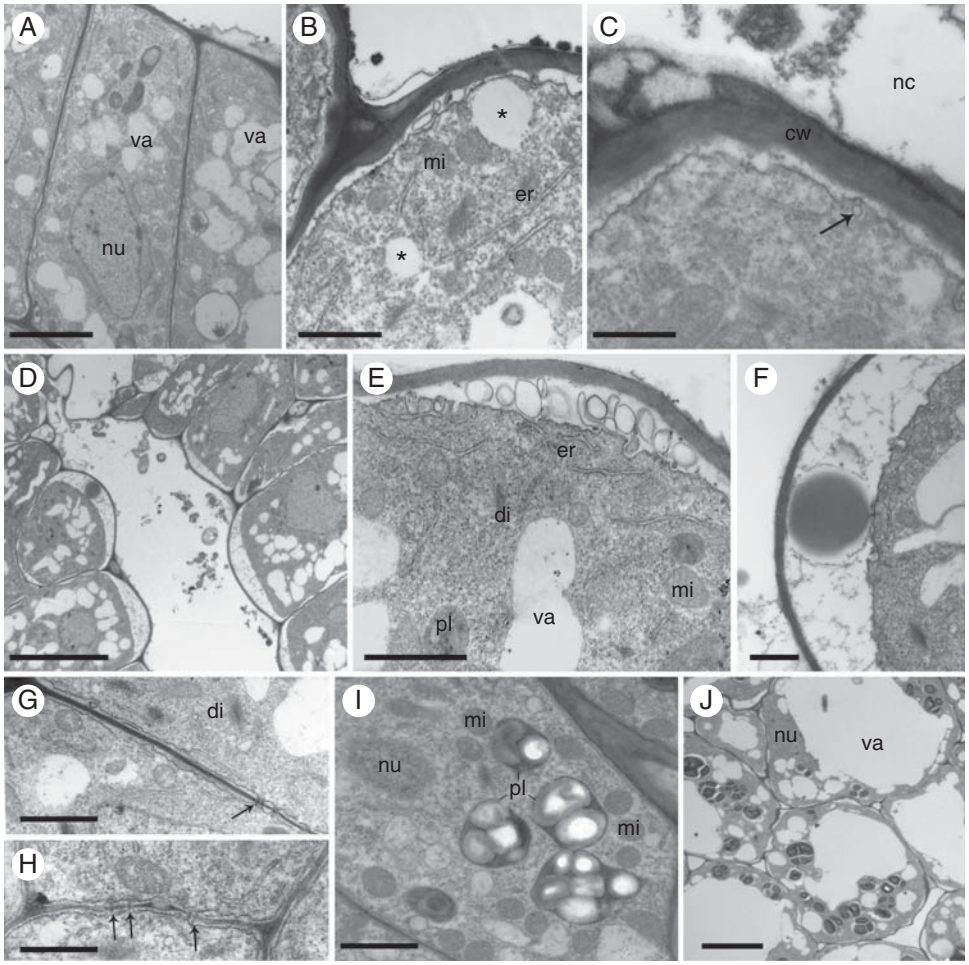

FIG. 3. Septal nectary ultrastructure of *Ananas ananassoides*. (A) Epithelial cells. nu, nucleus; va, vacuole. Note an electron-opaque layer, probably remnants of secretion, on the surface of the outer periclinal wall. Scale bar = 5 μm. (B) Part of two epithelial cells showing undulating plasma membrane, lipophilic drops (*), mitochondria (mi) and endoplasmic reticulum (er). Scale bar = 1 μm. (C) Translucent vesicles (arrow) near the plasma membrane. Note the absence of cuticle. Scale bar = 0·5 μm. (D) Part of a secreting nectary showing epithelial cells with ample periplasmatic space in their apical pole; note secretion residues in the channel. Scale bar = 10 μm. (E) Part of an epithelial cell with endoplasmic reticulum (er), dictyosome (di), mitochondria (mi), plastid (pl) and vacuoles (va). Note lamellar bodies in the periplasmatic space. Scale bar = 1 μm. (F) Part of an epithelial cell with flocculated material and a large lipophilic drop in the periplasmatic space. Scale bar = 0·7 μm. (G) Plasmodesmata (arrows) in the anticlinal walls of epithelial cells. Scale bar = 1 μm. (H) Plasmodesmata (arrows) in the inner periclinal walls of epithelial cells. Scale bar = 1 μm. (I) Part of an epithelial cell with nucleus (nu), plastids (pl) packed with prominent starch grains, and mitochondria (mi). Scale bar = 1 μm. (J) Nectary parenchyma cells showing nucleus (nu), reduced cytoplasm, plastids and one well-developed central vacuole (va). Scale bar = 7 μm.

Process of nectar secretion during anthesis and nectar sugar composition

'Visited' and 'unvisited' flowers presented a volume of nectar accumulated at 0600 h, of 23·47 ± 11·84 μL and 27 μl (median), respectively. In each group, 'visited' and 'unvisited', we registered just one flower with no nectar at 0600 h. Nectar volume in 'unvisited' flowers appeared to increase until 0800–0900 h, reaching a total of approx. 60 μL per flower (Fig. 4A). Between 0900 and 1300 h, nectar volume reached the maximum and remained almost constant (Fig. 4A). Thereafter, there was a continuous decrease in the total volume per flower, with a low amount of nectar per flower registered at 1600 h (Fig. 4A). For the 'visited' flowers there was a decrease in the nectar accumulated per hour during the afternoon in terms of both volume (Fig. 5A) and mass of sugar (Fig. 5B). Nectar concentration in 'visited' and 'unvisited flowers varied very little during anthesis (Fig. 6); thus, nectar solutes (milligrams of sugar, Figs 4B and 5B) showed the same pattern described for nectar volume, for both groups.

We found a general effect of repeated nectar removals on accumulated total production during a flower's life (Table 3). The mean total accumulated nectar volume of 'visited' flowers was higher than the mean volume in 'unvisited' flowers, and an inverse pattern was registered for nectar concentration values (Table 3). Thus, volume differences between flower groups can disappear in terms of nectar solutes since mean nectar concentration in the 'visited' and 'unvisited' flowers differed significantly. However, the differences were confirmed, because a higher quantity of nectar (in milligrams of sugar) was produced in the 'visited' flowers compared with 'unvisited' ones (Table 3). The nectar

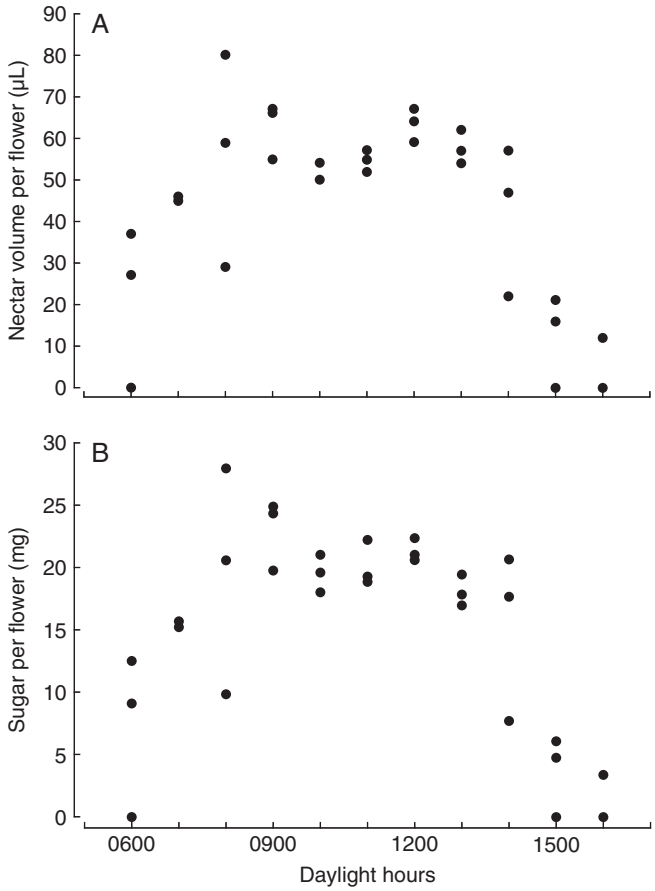

FIG. 4. Nectar secretion in 'unvisited' (control) flowers of *Ananas ananassoides* in a cerrado vegetation, Brazil ($n = 33$ flowers). (A) Nectar volume (μL). (B) Total mass of sugar (mg).

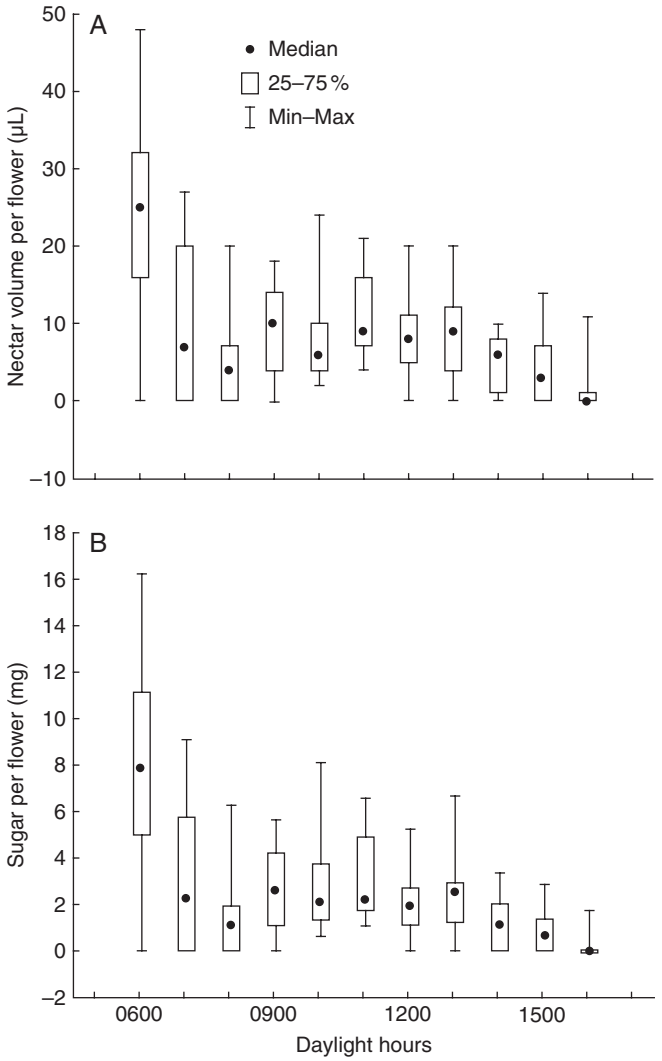

FIG. 5. Nectar secretion in 'visited' (nectar experimentally emptied) flowers of *Ananas ananassoides* in a cerrado vegetation, Brazil ($n = 17$ flowers). (A) Nectar volume (μL). (B) Total mass of sugar (mg).

carbohydrate composition for this species was $138{\cdot}9 \pm 27{\cdot}3$ mg mL^{-1} of sucrose, $61{\cdot}6 \pm 9{\cdot}9$ mg mL^{-1} of glucose and $52{\cdot}7 \pm 3{\cdot}5$ mg mL^{-1} of fructose, indicating a sucrose-dominated nectar.

DISCUSSION

Plant–pollinator interactions

The two hummingbird species that pollinate *A. ananassoides* are relatively small bodied and short beaked (Mendonça and Anjos, 2005), and they are important visitors of other Bromeliaceae species (Araújo and Sazima, 2003; Canela and Sazima, 2003; Kaehler *et al.*, 2005; Machado and Semir, 2006). Individuals of *Hylocharis chrysura* possess beaks approx. 19 mm long (Mendonça and Anjos, 2005), which is comparable with the dimensions of *A. ananassoides* flowers and with the distance between the base of the corolla tube and the floral reproductive structures. Machado and Semir (2006) showed that in dense populations of two bromeliads, *Aechmea nudicaulis* and *Vriesea philippocoburgii*, individuals of one hummingbird species, *T. glaucopis*, were the only visitors and exhibited territorial behaviour. In contrast, we observed that in sparser populations of *A. ananassoides*, *T. glaucopis* acted as a trapliner, exhibiting feeding behaviour similar to that

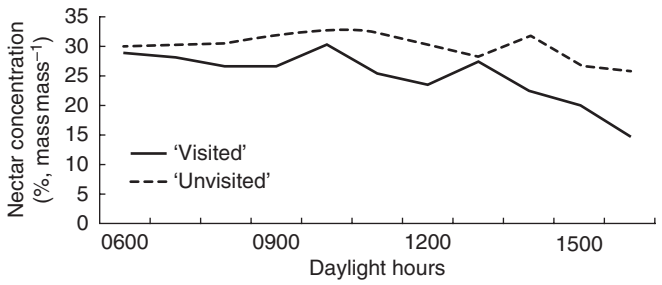

FIG. 6. Nectar concentration in 'unvisited' (control, $n = 33$ flowers) and 'visited' (nectar experimentally emptied, $n = 17$ flowers) flowers of *Ananas ananassoides* in a cerrado vegetation, Brazil.

observed by Canela and Sazima (2005) in *Bromelia antiacantha* (Bromeliaceae). Trapline foraging has been reported for various animal species collecting food from renewable resource patches (Ohashi and Thomson, 2009, and references therein). Considering that *A. ananassoides* is predominantly allogamous,

TABLE 3. *Comparison of mean values of* Ananas ananassoides *floral nectar using the* t*-test, in cerrado vegetation, Pratânia, SP, Brazil*

	Mean ± s.d.			
	'Visited' (drained flowers; $n = 17$)	Unvisited (bagged flowers; $n = 15$)	t	P
Volume (μL)	83·53 ± 20·28	57·67 ± 4·04	4·94	0·0001
Mass of sugar (mg)	27·80 ± 7·80	20·60 ± 4·00	2·95	0·007
Concentration (%, mass mass^{-1})	25·95 ± 4·83	30·46 ± 2·07	8·04	0·0001

with low self-fertility (0–8 %) (Coppens *et al.*, 1993), such trapline feeding behaviour favours outcrossing, which is particularly important for this bromeliad species.

Nectary structure and ultrastructure

Based on position, the septal nectaries of *A. ananassoides* are considered to be interlocular (*sensu* Simpson, 1998), as in other epigynous Bromelioideae investigated (Sajo *et al.*, 2004). The anatomy of the septal nectary of *A. ananassoides* agrees generally with previous reports for other Bromeliaceae species (Daumann, 1970; Cecchi Fiordi and Palandri, 1982; Varadarajan and Brown, 1988; Bernardello *et al.*, 1991), in which it was found that the septal nectaries are structural (cf. Fahn, 1979) because they are histologically differentiated into an epithelium and nectariferous parenchyma.

Our results showed that the septal nectary of *A. ananassoides* lacks a cuticle in the convolute region, which could be related to the mechanism of the channel development that occurs by schizogenesis (S. R. Machado *et al.*, unpubl. res.). The absence of cuticle in this region could not be considered as an artefact since we have registered the presence of intact cuticle in the nectary of other species processed by the same methods for optical and TEM analysis (Machado *et al.*, 2006, 2008; Paiva and Machado, 2008). Absence of cuticle in septal nectaries was also reported by Bernardello *et al.* (1991) for the bromeliad *Tillandsia tenuifolia*. Therefore, the secretion of the nectar in *A. ananassoides* seems to occur through the cell wall of epithelial cells. The overall ultrastructure of the septal nectary examined here is similar to that previously described in the nectar-producing tissues (Fahn, 1988; Nepi, 2007; Paiva and Machado, 2008). Abundance of mitochondria, primarily in the sub-epithelial layers of parenchyma cells, is consistent with elevated energetic demands due to secretory processes, and shows the involvement of this tissue in the secretion of nectar, as reported for other nectaries (Nepi, 2007). The septal nectary of *A. ananassoides* is not vascularized, but it is associated with numerous vascular bundles of the ovary; in addition, the nectary and vascular parenchyma cells contain large starch reserves that can used as energy in the pre-secretory and secretory phases, as commonly seen in the nectaries of other plants (Paiva and Machado, 2008). The presence of endoplasmic reticulum and vesicles close to the plasma membrane, and formation of ample periplasmatic spaces in the epithelial cells of *A. ananassoides*, suggest that the elimination of secretions from the protoplast is by exocytosis. This process of secretion has been observed in other glands of different species (Fahn, 1979, Nepi, 2007).

According to Ren *et al.* (2007), the sugar in nectar is supplied from at least two sources: the hydrolysis of nectary starch in the late phase of nectary development and the flux of photosynthates into the nectary. When a large amount of nectar is produced in a short time, it is generally produced from the hydrolysis of starch stored in the parenchyma (Pacini *et al.*, 2003), and this seems to be the case for *A. ananassoides*. Starch stored in nectary parenchyma could act as a finite resource in the short-lived flowers of *A. ananassoides* and could thus explain the lower concentration of nectar in repeatedly emptied flowers, which suggests a depletion of available carbohydrates in nectary parenchyma.

Process of nectar secretion during anthesis and nectar sugar composition

Our results for volume and concentration of floral nectar in *A. ananassoides* agree with the findings for other ornithophilous Bromeliaceae species reported by Galetto and Bernardello (2003), Canela and Sazima (2005) and Machado and Semir (2006). Hovering hummingbird pollinators have particular requirements as they have higher energy needs than bees, butterflies and other insects (Cruden *et al.*, 1983; Nicolson, 2006) and more need of water shunting than other species of birds (Nicolson, 2006), and their associated flowers usually produce copious nectar that is more dilute than that produced by insect-pollinated flowers (Baker and Baker, 1983). Mean nectar volume produced by a flower throughout its lifetime in the six Bromeliaceae species studied by Galetto and Bernardello (1992) was 16·4 ± 14·23 μL, and the mean amount of sugar produced per flower was 7·36 ± 6·93 mg. Flowers of these species lasted from 15 h to 6 d, exhibiting a much longer life span in general than *A. ananassoides* flowers. Nevertheless, despite their short life, *A. ananassoides* flowers presented a greater accumulated volume and mass of sugar during their life span, resulting in a considerable energy source for pollinators.

Nectar in ornithophilous flowers presents an average dilution of 75–80 % water (Nicolson and Fleming, 2003), which is comparable with the values verified in *A. ananassoides* floral nectar. Maintenance of the sugar concentration, even in dry warm environments, ensures adequate viscosity to allow nectar consumption by pollinators, which is especially important for nectarivorous birds (Baker, 1975; Nicolson, 1995; Nicolson and Nepi, 2005). In *A. ananassoides* flowers, nectar concentration, varying from approx. 26 % to 30 %, remained almost constant throughout anthesis, favouring collection and possibly fidelity by hummingbird pollinators. The concentration of nectar can be much more important for pollinators than the volume because it greatly affects nectar energy intake and osmotic balance, as well as their ability to collect it from the nectary or from the nectar chamber (Roberts, 1996). The sucrose-dominant nectar of *A. ananassoides* matches hummingbird preferences, as previously described for other ornithophilous species by Baker and Baker (1983), Stiles (1976) and Galetto and Bernardello (2003). A prevalence of sucrose in Bromeliaceae nectar and a convergence of nectar features in

hummingbird-visited species growing in different biogeographic regions were found by Galetto and Bernardello (2003).

In *A. ananassoides*, flowers repeatedly emptied of nectar clearly showed an increase in both the total volume and total milligrams of sugar produced. This result differs from that seen in other species of Bromeliaceae, in which the total sugar production was not affected by repeated nectar removal or was diminished, as in *Puya spathacea* (Galetto and Bernardello, 1992). The *A. ananassoides* septal nectary corresponds to the 'labyrinthine common nectarial cavity' type, *sensu* Schimd (1985), which increases the nectary surface by undulation and convolution as observed by Bernardello *et al.* (1991) in other Bromeliaceae species. The continuous nectar production throughout the day in repeatedly 'visited', i.e. experimentally emptied, flowers of *A. ananassoides* may be associated with the structure of the nectary that has a great longitudinal size, a labyrinthine surface, phloem and abundant xylem. Therefore, these nectary features could allow a rapid nectar renewal after a visit and a relatively constant supply of water and solutes for traplining hummingbirds throughout the day.

Commonly, hummingbird-pollinated flowers begin to secrete nectar 1–4 h prior to the activity of its pollinators, and the rate of secretion continues until some critical amount has accumulated, and then nectar secretion ceases (Stiles, 1976; Cruden *et al.*, 1983; Castellanos *et al.*, 2002), similar to the pattern verified in *A. ananassoides* flowers. Based on our observations, three different phases of the process of secretion throughout anthesis could be hypothesized, comprising periods of active secretion, cessation and reabsorption of nectar. A short initial secretion period was identified in 'unvisited' flowers of *A. ananassoides*, as well as a clear decrease in the water and sugar content after 1400 h, suggesting the occurrence of active nectar reabsorption. This nectar decrease was not due to evaporation, because nectar concentration was maintained during the whole of anthesis. Usually, the concentration of nectar increases and its volume decreases, as a consequence of evaporation (Cruden *et al.*, 1983). Reabsorption of nectar has been assumed to have the function of retrieving energetically valuable sugars that are not utilized by pollinators (Nicolson, 1995). Considering that nectar production can require a high energy investment (Southwick, 1984), the recycling of sugar not collected by floral visitors could represent an important mechanism of energy saving by the plant, as the expenditure of energy in the reabsorption process could be lower than the energy recovered by sugar influx, resulting in an overall net gain, as reported by Stipczynska and Nepi (2006). Nectar reabsorption in *A. ananassoides* flowers may be related to the large surface area of the septal nectary. It is important to note that the contact of nectary tissue with the secreted nectar is a prerequisite for nectar reabsorption (Bonnier, 1878; Búrquez and Corbet, 1991; Nepi *et al.*, 1996). For *A. ananassoides*, in the absence of pollinator visits, the reabsorption of water and sugar could represent an important mode of energy saving, as this species produces a fleshy fruit clustered into a highly hydrated, sweet infructescence. Nevertheless, available nectar may be close to zero if visitation by pollinators to open flowers is intense; then reabsorption may not be energetically significant to the plant. However, considering that visitation rates of pollinators can vary between days, populations and among plants of a population, this ability of *A. ananassoides* could represent an advantage in some situations.

In contrast, the process of nectar secretion was maintained until 1600 h if flowers were repeatedly 'experimentally emptied' of nectar every hour ('visited' flowers), indicating that secretion ability is preserved throughout the flower's life span if visits of pollinators take place. These results suggest that if flowers of *A. ananassoides* are not visited, a homeostatic mechanism drives active nectar reabsorption; this physiological mechanism that adjusts nectar solutes and volume was previously reported for other plant species (Galetto *et al.*, 1994; Nepi *et al.*, 2011). Thus, the process of nectar secretion can be adjusted, favouring successive visits, i.e. renewal of nectar after a visit; or a nectar-saving mechanism can be activated if flowers are not visited, i.e. active secretion is diminished and nectar reabsorption occurs at the end of the flower's lifetime.

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