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Nectar secretion dynamic links pollinator behavior to consequences for plant reproductive success in the ornithophilous mistletoe *Psittacanthus robustus*

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ABSTRACT

The mistletoe *Psittacanthus robustus* was studied as a model to link flower phenology and nectar secretion strategy to pollinator behaviour and the reproductive consequences for the plant. The bright-coloured flowers presented diurnal anthesis, opened asynchronously throughout the rainy season and produced copious dilute nectar as the main reward for pollinators. Most nectar was secreted just after flower opening, with little sugar replenishment after experimental removals. During the second day of anthesis in bagged flowers, the flowers quickly reabsorbed the offered nectar. Low values of nectar standing crop recorded in open flowers can be linked with high visitation rates by bird pollinators. Eight hummingbirds and two passerines were observed as potential pollinators. The most frequent flower visitors were the hummingbirds *Eupetomena macroura* and *Colibri serrirostris*, which actively defended flowering mistletoes. The spatial separation between anthers, stigma and nectar chamber promotes pollen deposition on flapping wings of hovering hummingbirds that usually probe many flowers per visit. Seed set did not differ between hand-, self- and cross-pollinated flowers, but these treatments set significantly more seeds than flowers naturally exposed to flower visitors. We suggest that the limitation observed in the reproductive success of this plant is not related to pollinator scarcity, but probably to the extreme frequency of visitation by territorial hummingbirds. We conclude that the costs and benefits of plant reproduction depend on the interaction strength between flowers and pollinators, and the assessment of nectar secretion dynamics, pollinator behaviour and plant breeding system allows clarification of the complexity of such associations.

INTRODUCTION

Nectar production patterns during anthesis are diverse, and such variability is related to environmental, pollinator and plant traits (Cruden *et al.* 1983; Boose 1997; Ornelas *et al.* 2007). For example, individually or combined, intrinsic morpho-physiological plant traits (flower and nectary size, mode of nectar synthesis and secretion, floral longevity and phenology, resorption capacity) and extrinsic traits (pollinator behaviour, air temperature and humidity, nectar robbers) are related to differences in the nectar secretion patterns, which affect nectar standing crop (e.g. Pleasants & Chaplin 1983; Torres & Galetto 1998; Lara & Ornelas 2002; Galetto & Bernardello 2004). In addition, nectar resorption has been assumed to have the function of retrieving energetically valuable sugars that are not used by pollinators (Cruden *et al.* 1983; Búrquez & Corbet 1991; Rivera *et al.* 1996; Stahl *et al.* 2012). Nectar production dynamics can be analysed during flower anthesis, because nectar is secreted in particular rhythms according to both plant characteristics and behaviour of pollinators. The main physiological mechanism that adjusts nectar traits has previously been reported for other plant species (Galetto *et al.* 1994; Castellanos *et al.* 2002; Nepi *et al.* 2011; Veiga *et al.* 2013). Thus, the pattern of nectar secretion can be selected, favouring, or not, successive visits by pollinators in order to increase plant reproductive success (Mitchell & Waser 1992; Boose 1997).

Knowledge of nectar production and availability is essential for analysis of pollinator behaviour (Gill & Wolf 1975; Rathcke 1992). Without such knowledge, aspects such as the plant's strategy of offering nectar, the activity patterns of floral visitors and the rate of nectar consumption by animals, among others, cannot be clearly understood (Galetto & Bernardello 2004; Galetto & Bernadello 2005; Agostini *et al.* 2011). For example, some ornithophilous species present nectar replenishment after a hummingbird visit, whereas other species do not (Galetto & Bernardello 1992; Ordano & Ornelas 2004, 2005; Stahl *et al.* 2012); hummingbirds need specific nectar amounts as they have high energy requirements (Cruden *et al.* 1983; Johnson & Nicolson 2008), which generally determine fidelity to a particular nectar source (Gill & Wolf 1975). Enhanced attraction of pollinators favours seed production if pollination is sufficient to fertilise the available ovules of each individual (Barrett & Harder 1996). Therefore, nectar traits and nectar secretion patterns may be related to the frequency of visits to an individual plant and also to each flower, with consequences for reproductive success *via* pollen deposition and delivery (Mitchell & Waser 1992; Rathcke 1992). Conversely, the intensity of visitation by pollinators may affect the amounts of nectar replenished or reabsorbed in flowers, with negative consequences for plant reproduction related to trade-offs between energy allocated to nectar secretion and seed production (Pyke 1991; Ordano & Ornelas 2005; Ornelas & Lara 2009).

Mistletoes comprise a special group of parasitic plants that are a keystone resource in forests and woodlands worldwide (Watson 2001). These plants usually present extended flowering and fruiting phenologies, production of high-quality nectar and fruits, and few chemical or structural defences, being widely used by vertebrates as food and thus positively affecting alpha diversity (Watson 2001). Among mistletoes, Loranthaceae is the largest family, with about 990 species within 73 genera (Nickrent *et al.* 2010), and many species present a set of traits related to pollination by nectarivorous birds (Kuijt 1969). Most studies reported mistletoe floral nectar as the main reward for pollinating birds in Africa (Gill & Wolf 1975; Feehan 1985), Asia (Docters van Leeuwen 1954; Davidar 1985), Australia (Ford *et al.* 1979; Bernhardt & Calder 1981), New Zealand (Ladley *et al.* 1997; Robertson *et al.* 1999) and the Americas (Feinsinger 1978; Graves 1982; Galetto *et al.* 1990; Medel *et al.* 2002; Aizen 2003). Some mistletoe species are totally dependent on bird pollination for reproduction (Rivera *et al.* 1996; Robertson *et al.* 1999), while other species are able to produce seeds spontaneously through autonomous self-pollination (Ladley *et al.* 1997; Ramírez & Ornelas 2010) or by apomixis (Medel *et al.* 2002). However, the relationship among nectar secretion dynamics and visitation behaviour of pollinators and the consequences for plant reproduction remains poorly understood for ornithophilous mistletoes.

The Neotropics harbour the highest diversity of mistletoes (Calder & Bernhardt 1983; Nickrent *et al.* 2010), but the nature of mutualistic interactions between mistletoes and birds are still poorly investigated in tropical ecosystems of the New World (Azpetia & Lara 2006). *Psittacanthus* Mart. comprises the most diversified genus within Loranthaceae, with nearly 120 species distributed from Mexico to South America (Kuijt 2009; Nickrent *et al.* 2010). Most species in the genus are recognised as ornithophilous (Feinsinger 1978; Buzato *et al.* 2000; Vasconcelos & Lombardi 2001; Araújo & Sazima 2003; Azpetia & Lara 2006; Leal *et al.* 2006; Rocca & Sazima 2008), except for *Psittacanthus acinarius* Mart. and *P. corynocephalus* Eichler, which are considered to be chiropterophilous (Araújo & Sazima 2003; Fleming *et al.* 2009). However, data on pollination ecology are restricted to *P. calyculatus* (DC.) G. Don (Azpetia & Lara 2006) or to some observations on nectar secretion and breeding system for *P. schiedeanus* (Schlecht. & Cham.) G. Don (Ramírez & Ornelas 2010), both considered ornithophilous species.

In this study, we investigated the pollination ecology of the mistletoe *P. robustus* Mart. (Loranthaceae) in a highland savanna site comprising quartzite rocky outcrops at the Serra do Cipó mountains, southeastern Brazil. We used this plant species as a model to link flower traits (e.g. phenology, nectar secretion strategy, removal effects on total nectar production), pollinator behaviour and the reproductive consequences for the plant. In this sense, we hypothesised that nectar secretion dynamics would favour successive visits by hummingbird pollinators through flower anthesis, enhancing reproductive success of this mistletoe species. Therefore, we predicted that flowers naturally exposed to hummingbirds would set more seed than flowers excluded from visits of pollinators. In order to evaluate these predictions, we obtained data on (i) floral biology and phenology; (ii) nectar secretion dynamics during flower anthesis and the effects of nectar removals on total nectar production; (iii) behaviour and patterns of visits of the main pollinators; and (iv) their pollination effectiveness on plant fruit set.

MATERIAL AND METHODS

Study area

We conducted the study in a specific protected highland savanna area, comprising quartzite rocky outcrops varying from 1100 to 1300 m a.s.l. in the vicinity of Serra do Cipó National Park, Minas Gerais, southeastern Brazil (43°33′W, 19°15′S). The Serra do Cipó Mountains are situated in the southern portion of the Espinhaço Range, a predominantly quartzite mountain range that extends for 1100 km from southeastern to northeastern Brazil and is closely related to the cerrado biome along its western slopes. This region supports many endemic and threatened plant species (Giulietti *et al.* 1997), comprising a region of extreme importance for biodiversity conservation in Brazil (Echternacht *et al.* 2011). Rocky outcrop ecosystems are associated with quartzite rocks having shallow, acid, sandy and nutrient-poor soils (Ribeiro & Fernandes 2000). Climate is mesothermic (Cwb of Köppen), with cold dry winters from May to September and hot wet summers from October to April, and a mean monthly temperature varying from 17 to 23 °C. Average monthly precipitation varies from 11.9 mm in July to 281.1 mm in January, with annual precipitation around 1400 mm (Madeira & Fernandes 1999).

Phenology and floral biology

In September 2007 we selected 47 mistletoes, 15 of which parasitise *Vochysia thyrsoidea* Pohl, 15 *Qualea cordata* (Mart.) Warm (Vochysiaceae) and 17 *Trembleya laniflora* Triana (Melastomataceae). For each mistletoe plant, we recorded the number of buds and open flowers twice a month, every 15 days, until the end of the flowering season in March 2008. Flowers (n = 47; one per mistletoe from distinct hosts) were collected during January 2009 to characterise floral biology. Flower parts were measured using calipers (error: 0.05 mm). To determine the number of flowers per inflorescence, we counted the number of flower buds on 85 randomly selected inflorescences, one per mistletoe. To evaluate floral longevity, we conducted daily inspections following 72 tagged buds in eight mistletoes during January 2008. We also recorded other flower features, such as floral opening time, colour, odour, pollen availability on stamens and stigma appearance.

Nectar secretion pattern, removal effects on total production and standing crop

In January 2009 we conducted an experiment to examine nectar secretion dynamics on seven mistletoes parasitising distinct *Vochysia thyrsoidea* tree hosts. All trees were exposed to similar climate conditions because they were located within a set area (100-m radius). Our experimental design followed standard procedures proposed in Galetto & Bernadello (2005), which allowed the determination of nectar secretion rates and the effects of removal on overall reward availability. We extracted nectar samples without removing flowers, always carefully inserting glass capillary tubes so as to avoid damaging flowers and nectaries. Two variables were measured immediately: nectar volume (μl) and sugar concentration (% mass sugar/total mass solution) using a pocket refractometer (BTX-1 Vee Gee Brix, range 0–32%). In order to estimate energy values, it was necessary to convert mass percentages to mass-based concentrations. We used the formula: $Y = 0.00226 + (0.00937X) + (0.000585X^2)$ according to Galetto & Bernadello (2005), where (Y) represents the nectar sugar concentration (mg μl^{-1}) for a given sugar mass percentage (X) determined with the refractometer. The amount of sugar available from a flower could then be calculated by multiplying the nectar sugar concentration by the volume of nectar. We tagged flower buds individually, and bagged inflorescences with mesh bags to prevent nectar removal by flower visitors. Over 1 week, we followed flower bud opening, and established nine treatments (sets) of nectar removal at different periods of the flowers' lifetime, using one flower per treatment from each mistletoe plant (n = 7). Our experimental design allowed comparison among treatments (sets) within the same plants. In set-I, flowers were sampled between 07:00–08:30 h on the first day of anthesis, and then sampled again at midday (12:00–13:30 h and in the afternoon (17:00–18:30 h), repeating the same schedule in the next 2 days of anthesis, with a total of nine nectar samples. The following treatments (sets-II–IX) consisted of sampling nectar for the first time, one period later than the previous treatment according to the scheme provided in Table 1. Therefore, flowers in set-II were sampled for the first time at midday of the first day and on seven more occasions, and so on, until set-IX with flowers being sampled only once in the afternoon of the third day, *ca*. 59 h after flower opening. Inflorescences remained bagged between nectar removals. Total accumulated nectar (volume and sugar mass) corresponded to the sum of all nectar samples obtained for each flower.

Nectar standing crop was evaluated, measuring the volume and concentration of the available nectar (volume, concentration and mg sugar flower^{-1}) from 67 open flowers that had been exposed to pollinators. Data were collected at random for individual flowers at three different periods of the day (08:00–09:00, 11:00–13:00 and 16:00–18:00 h), including all flower ages.

Plant breeding system

To evaluate the breeding system of *P. robustus*, we conducted an experiment following standard procedures according to Dafni (1992). In January and February 2008 we tagged 28 mistletoe plants parasitising distinct *V. thyrsoidea* trees in an area of *ca*. 10 ha. We selected 10–18 accessible inflorescences per mistletoe, removing all open flowers and developing fruits, tagging flower buds individually and then isolating inflorescences with mesh bags (<1 mm mesh). For each treatment, we used 17–30 flowers per plant. In 14 mistletoes, we simply bagged inflorescences to exclude flower visitors (autonomous self-pollination treatment, n = 406 flowers) and the remaining inflorescences remained accessible to flower visitors to evaluate natural fruit set (control treatment, n = 420). In another 14 mistletoes we applied three distinct treatments to each plant. Initially, we set adhesive tape on the bud tip to prevent corolla opening during anthesis, then we emasculated the flower by cutting the corolla laterally and clipping the stamens to prevent any contact between pollen grains and the stigma. We then hand-cross-pollinated flowers by brushing six to seven anthers against the stigmas of flowers over a period of 2–3 days. In the xenogamous pollination treatment (n = 333) we used stamens collected from mistletoes at least 100 m from the recipient plant, and in the geitonogamous pollination treatment (n = 316) we used stamens collected in distinct flowers from the same mistletoe. In the apomixis treatment (n = 420), we clipped the stigma at the mid-section and blocked it with water based glue. After applying these treatments we bagged inflorescences again. We evaluated final fruit set in April 2008, approximately 3 months after the beginning of the experiments. Fruits were harvested, sliced longitudinally and checked for the presence of seeds. Each flower developed into one fruit with a single seed, thus we determined seed set as fruit/flower ratio in each treatment. We considered seed set in each plant as a replicate for distinct treatments. We calculated the index of self-incompatibility (ISI) by dividing self-pollination seed set by the xenogamous pollination seed set following Aizen (2005), with values close to 1 (>0.9) indicating full self-compatibility.

Behaviour, visitation rate and abundance of flower visitors

To determine frequency and behaviour of flower visitors, we performed 120 h of focal observations over 15 days, distributed throughout the flowering season from December 2007 to February 2008. We selected 40 large conspicuous flowering mistletoes along a 2-km transect. Each plant was observed at three different periods of the day: in the morning (07:00–10:00 h), at midday (11:00–14:00 h) and in the afternoon (15:00–18:00 h), always on different days. Each observation period lasted 1 h, and after that we moved to another plant located at least 50 m apart. We made observation bouts throughout the whole day from 06:30 h to 18:30 h, observing seven to nine different plants per day using binoculars (8 × 23 Canon) at distances varying from 5 to 10 m. During each observation bout we recorded: (i) identity of the flower visitor, (ii) number of visits, defined as the approach with consumption of nectar from at least one flower, (iii) number of flowers probed per visit, (iv) foraging mode (perched or hovering), and (v) body parts contacting reproductive structures of flowers. We also recorded agonistic encounters between flower visitors. Visitation rate is expressed as the mean number of visits per plant in 1 h (visit.plant^{-1} h). Data were recorded for each visitor species throughout the entire flowering season.

We determined relative abundance of different hummingbird species at the study site using the point transect method (Gregory *et al.* 2004). We established 10 points set 200 m apart from each other along the same 2-km trail used in focal

Table 1. Nectar secretion dynamics throughout flower anthesis of seven individuals of *Psittacanthus robustus* growing on *Vochysia thyrsoidea* in a highland rocky savanna in the Serra do Cipó Mountains, southeastern Brazil.

day	1			2			3		
T O D	morning	midday	afternoon	morning	midday	afternoon	morning	midday	afternoon
HAO	1	6	11	25	30	35	49	54	59
set I									
vol (μl)	118.5 ± 55.1	19.7 ± 13.7	27.2 ± 19.4	57.8 ± 64.6	0	0	0	0	0
sugar (%)	12.8 ± 2.1	10.3 ± 1.4	8.5 ± 4.3	2.6 ± 2.8	0	0	0	0	0
sugar (mg)	15.5 ± 7.5	2.1 ± 1.6	2.9 ± 2.4	2.0 ± 3.5	0	0	0	0	0
set II									
vol (μl)		65.7 ± 31.7	28.0 ± 28.6	35.3 ± 52.3	0.9 ± 2.5	0	0	0	0
sugar (%)		16.3 ± 5.3	9.5 ± 6.6	4.2 ± 4.4	0.5 ± 1.5	0	0	0	0
sugar (mg)		12.4 ± 8.7	3.5 ± 3.6	1.6 ± 2.2	0.0 ± 0.1	0	0	0	0
set III									
vol (μl)			68.7 ± 43.2	28.4 ± 27.7	0.4 ± 1.1	0	0	0	0
sugar (%)			15.7 ± 6.1	5.2 ± 4.4	1.0 ± 2.9	0	0	0	0
sugar (mg)			11.7 ± 7.9	2.3 ± 2.2	0.0 ± 0.1	0	0	0	0
set IV									
vol (μl)				87.6 ± 64.0	0.6 ± 1.1	0	0	0	0
sugar (%)				9.3 ± 5.8	6.9 ± 19.9	0	0	0	0
sugar (mg)				10.6 ± 11.1	0.0 ± 0.1	0	0	0	0
set V									
vol (μl)					19.3 ± 26.5	0	0	0	0
sugar (%)					7.9 ± 8.2	0	0	0	0
sugar (mg)					2.2 ± 2.8	0	0	0	0
set VI									
vol (μl)						23.7 ± 29.8	0	0	0
sugar (%)						10.1 ± 11.7	0	0	0
sugar (mg)						4.4 ± 5.4	0	0	0
set VII									
vol (μl)							6.5 ± 17.1	0	0
sugar (%)							1.6 ± 4.2	0	0
sugar (mg)							0.7 ± 1.9	0	0
set VIII									
vol (μl)								7.7 ± 13.1	0
sugar (%)								4.0 ± 6.9	0
sugar (mg)								1.1 ± 1.9	0
set IX									
vol (μl)									6.2 ± 16.4
sugar (%)									0.1 ± 0.4
sugar (mg)									0.07 ± 0.2

TOD, time of day; HAO, hours after flower opening, approximate values; Vol, nectar volume in μl; Sugar (%), sucrose concentration; Sugar (mg), sucrose mass in milligrams.

observations. From December 2007 to February 2008 we performed 10 hummingbird counts, which consisted of visiting all points sequentially from 06:30 to 10:00 h. We spent 10 min in each point, recording the number of hummingbirds seen or heard within a 50-m radius. An index of relative abundance was calculated by dividing absolute number of records for each species by the total number of point counts (n = 100). We also used published data on hummingbird abundance obtained with mist-net sampling procedures, a total effort of 72,551 h m^{-2} of mist-nets, over 45 days during 14 months in the study site (for details see Costa & Rodrigues 2012).

Data analyses

We compared nectar volume and sugar mass among different treatments using randomised block ANOVA after log transformations, using plants as the blocking factor. We also used randomised block ANOVA with plants as the blocking factor to compare total accumulated nectar volume and sugar mass (the sum of all samples) among sets within each plant. We only used sets I, II, III and IV for data analysis because flowers on the remaining treatments reabsorbed nectar. We also performed *post hoc* comparisons among treatments using Fisher LSD test. Standing crops (volume and amount of sugar per flower) between different periods of the day were compared using a Kruskal–Wallis test. We compared seed set among hand-pollination treatments within the same plant using two-tailed paired Student *t*-tests, and among treatments in different plants using two-tailed Student *t*-tests for independent samples, on arcsine square root-transformed data. We compared number of flowers probed per visit among hummingbird species using Kruskal–Wallis test and *post hoc* comparisons among species using Mann–Whitney tests. We compared observed frequency of visitation by each species of hummingbird (total number of visits observed during focal observations) to expected relative abundance (total number of records during point counts) of each species using the goodness-of-fit G test, considering only data for the five most abundant species in the analysis. All analyses were performed following Zar (1999), with $\alpha = 0.05$.

RESULTS

Phenology and floral biology

Psittacanthus robustus bears terminal and axillary umbellate inflorescences, with flower buds usually disposed in triads, occasionally in diads, but varying highly in number among inflorescences (mean ± SD, 7.5 ± 0.3, range 3–17, n = 85). The development of flower buds started in October and lasted until February (Fig. 1a). According to plant size, individuals may produce 300–600 flower buds over the flowering period (Fig. 1a). The flowering period was extended through the wet season. Open flowers were recorded from November to March, with a flowering peak at the end of December (Fig. 1b). Flowering was slightly asynchronous among individuals within the population. Although few large plants presented more than 40 open flowers per day during the flowering peak, most plants typically presented few simultaneously flowers in anthesis, usually <10 newly open flowers per day (Fig. 1b).

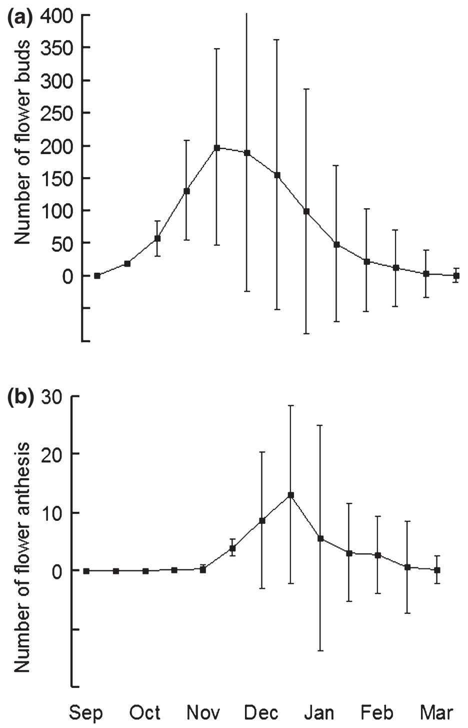

Fig. 1. Flowering phenology of *Psittacanthus robustus* at the highland rocky savanna site, southeastern Brazil. Points represent mean and whiskers SD of the number of flower buds (a) and flower anthesis (b) per plant, counted twice a month in 46 individual mistletoe plants.

Flowers usually opened sequentially within the same inflorescence. Flowers are hermaphroditic, the calyx is reduced to a uniform calyculus of nearly 1 mm in length and petals are 10–12-cm long, forming a hexamerous actinomorphic corolla. Petals detach at the extremity or in the middle, always forming a spiral corolla, but remain fixed at the proximal portion, forming an upward-oriented tubular nectar chamber 2–3-cm long. The nectaries are located at the base of the style in the receptacle (width 0.42 ± 0.03 cm, length 0.54 ± 0.06 cm, n = 50), and the ovary is inferior. Six stamens are attached to the middle of each petal through very long thin filaments (length 5.09 ± 0.61 cm, range 3.7–6.5, n = 50) with anthers (length 0.54 ± 0.09 cm, range 0.37– 0.81, n = 50) at the tips. The style is cylindrical and longer (length 10.01 ± 0.86 cm, range 7.5–12.5, n = 50) than the stamens, and the stigma is capitate.

Anthesis begins between 05:00 and 07:00 h, and the flowers last for about 3 days (3.1 ± 0.3 days, range 3–4, n = 72). On the first day the corolla is bright yellow, but petals become orange on the second day, pale orange on the third day, and wilt at the end of the third to fourth day. Petals present a pleasant but weak scent not found on the leaves. Anthers become dehiscent about 1 h after flower opening, and pollen remains attached to thecae for 2 days in bagged flowers. However, most pollen is removed within the first day in anthers exposed to pollinators. Pendant styles remain attached to receptacles for nearly 2 weeks after corolla abscission. During anthesis, the style remains erect whereas the filaments become diagonally positioned with a remarkable spatial gap between stigma and anthers.

Patterns of nectar secretion, removal effects on total nectar production and standing crop

Psittacanthus robustus flowers produced copious dilute nectar as the only reward for pollinators. Data reported in Table 1 describe the natural nectar secretion pattern of unvisited flowers (bagged) of *P. robustus* throughout flower anthesis. The amount of nectar per flower (measured in volume or sugar mass) was highest at the beginning of anthesis (mean *ca.* 120 μl or 15.5 mg flower^{-1}, respectively). After which there was a constant decrease in the amount of nectar (mg sugars) as the flower ages (Table 1). During the first day, an increase in nectar concentration (from 12.8% to 16.0%) and a decrease in nectar volume (from *ca.* 120 to ≤70 μl) was recorded, but the total amount of sugar per flower decreased (Table 1); this trend was continued during days 2 and 3 of anthesis (Table 1). It is interesting to note that the mean total volume per flower was higher during the second morning of the flowering (87.6 μl) than at the last measurement on the previous day (Table 1). Nevertheless, total amount of sugar per flower and nectar concentration were lower during the second day of flowering (Table 1). Thereafter all nectar parameters experienced a constant decrease until the end of flowering (Table 1). In summary, the active nectar secretion period occurs before flower opening and the amount of sugar was maintained in bagged flowers until the morning of the second day, after which flowers actively reduced nectar amount per flower (Table 1).

Experimental removal effects on total nectar production showed that nectar volume varied significantly among treatments (randomised block ANOVA, $F_{8,63} = 7.2$, $P < 0.001$), but not among plants (block effect: $F_{6,63} = 0.36$, $P = 0.89$). *Post-hoc* tests indicated that accumulated nectar volume on set-I was higher than on set-II but did not differ from sets-III and –IV; these four sets accumulated more nectar than the remaining sets (Fig. 2a). The amount of sugars also differed among sets subjected to a different number of removals (randomized block ANOVA, $F_{8,63} = 7.4$, $P < 0.001$), but also among plants ($F_{6,63} = 2.6$, $P = 0.02$). *Post-hoc* tests indicated that sugar mass did not differ significantly among sets-I to -IV, being higher than that of the remaining sets (V–VIII).

Because flowers actively reduced nectar after 24 h from the beginning of anthesis, removals effects on cumulative nectar production throughout flower anthesis were analysed by comparing only set-I to -IV. Total accumulated nectar volume secreted in flowers differed significantly among treatments (randomised block ANOVA: $F_{3,21} = 5.1$, $P < 0.001$; Fig. 2a), but not significantly among plants (block effect: $F_{6,21} = 2.2$, $P = 0.08$). *Post-hoc* tests indicated that nectar volume was higher in set-I than in the other three sets (but no statistically significant difference among them). The same trend was observed for total sugar mass production (means 22.5, 17.5, 14.0 and 10.6 mg for set-I, -II, -III and –IV, respectively; Fig. 2b). However, differences in total sugar mass secreted per flower among sets were not statistically significant ($F_{3,21} = 2.1$, $P = 0.13$; Fig. 2b). Although flowers can replenish nectar after removals, successive replenishments were lower and even more diluted (Table 1).

Nectar standing crop per flower was very low throughout the day in terms of volume (morning: 3.9 ± 4.3 μl, midday: 3.5 ± 3.8 μl and afternoon: 13.1 ± 26.3 μl) or amount of sugars (morning: 0.34 ± 0.39 mg, midday: 0.44 ± 0.49 mg and afternoon: 1.59 ± 3.34 mg). The differences in nectar standing crop among these periods were not statistically significant (H = 0.08, $P = 0.96$, H = 0.11, $P = 0.95$ for nectar volume and mg sugar flower^{-1}, respectively). Mean nectar standing crop per flower represented <6% of the natural nectar secreted in bagged flowers at the beginning of anthesis; 37% of open flowers evaluated (n = 67) presented no rewards for pollinators.

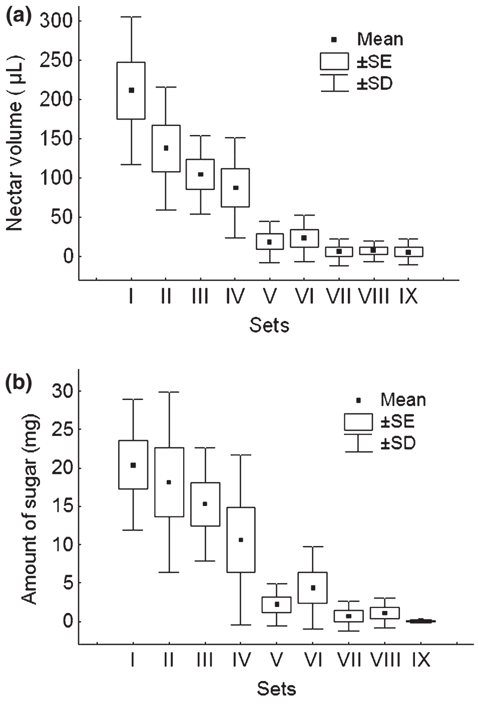

Fig. 2. Total volume of nectar (a) and total amount of sugar (b) produced throughout the flower lifetime in nine sets (I–IX) varying in number of removals (see Table 1).

Plant breeding system

Flowers did not set seed apomictically, but in experimental manual treatments involving pollen transference seed was set (Fig. 3). Mean seed set in the xenogamous (58%) did not differ from the geitonogamous (53%) treatment (paired *t*-test = 1.04, df = 13, $P = 0.31$). Seed set in the xenogamous pollination treatment was significantly higher than in the natural control treatment (t = 2.7, df = 13, $P = 0.01$) or autonomous self-pollinated flowers (t = 4.7, df = 13, $P < 0.0001$). Similarly, seed set in the geitonogamous treatment was also significantly higher than fruit set in the control (t = 2.2, df = 13, $P = 0.02$) or autonomous self-pollinated (t = 4.6, df = 13, $P < 0.001$) flowers. Mean seed set in natural-pollinated (control) flowers (40%) was significantly higher (paired *t*-test = 7.7, df = 13, $P < 0.001$) than in autonomous self-pollinated flowers (28%). The obtained ISI index was 0.91.

Behaviour, visitation rate and abundance of pollinators

We recorded 145 visits from 10 bird species in a period of 120 h of focal observations. The number of visits plant^{-1} h^{-1} varied from 0 to 7 (1.2 ± 1.4, range 0–6). Hummingbirds were the main group of visitors of *P. robustus*, responsible for 96% of visits (Table 2). To obtain nectar, they probed flowers by inserting their bill inside the corolla tube, always touching anthers and stigmas with their flapping wings and belly when hovering. Some species occasionally perched on flower buds to obtain nectar (Table 2). The mean number of flowers probed per visit varied among the five most frequent hummingbird species but differences were not statistically significant ($H_{4,117} = 4.48$, $P = 0.34$; Table 2). Hummingbirds usually probed many open flowers of an individual (mean 5–14 flowers per visit, according to species; see Table 2).

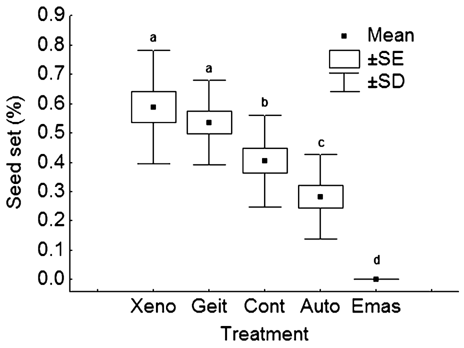

Fig. 3. Seed set (no. seeds/ no. flowers) in five pollination treatments: Xeno, hand-cross-pollination; Geit, geitonogamous hand-pollination; Cont, control flowers exposed to pollinators; Auto, autonomous self-pollination in bagged inflorescences; Emas, emasculation and apomixis. Different letters denote significant statistical differences among treatments.

Table 2. Visitation frequency, abundance and number of flowers probed by potential bird pollinators of *Psittacanthus robustus* (Loranthaceae) in a highland rocky savanna, southern Espinhaço Range, Brazil.

					NFP			
bird species	NV (%)	VR	IRA	CAP (%)	N	mean	±SD	range
Trochilidae								
Eupetomena macroura[a,b] (Gmelin, 1788)	88 (60.6)	0.733	0.16	3 (2.5)	77	4.7	3.3	1–15
Colibri serrirostris[a,b] (Vieillot, 1816)	24 (15.5)	0.200	0.06	21 (17.5)	21	8.7	7.9	1–30
Phaethornis pretrei[a,b] (Lesson & Delattre, 1839)	9 (6.2)	0.075	0.11	12 (10)	5	5.8	3.8	3–12
Augastes scutatus[a] (Temminck, 1824)	8 (5.5)	0.066	0.36	67 (55.8)	6	5.1	4.2	1–12
Chlorostilbon lucidus[a] (Shaw, 1812)	7 (4.8)	0.058	0.05	15 (12.5)	5	4.8	1.3	3–6
Heliactin bilophus[a] (Temminck, 1820)	2 (1.3)	0.016	0.01	1 (0.83)	2	14.0	2.8	12–16
Calliphlox amethystina[a] (Boddaert, 1783)	1 (0.6)	0.008	0	0	1	10	–	–
Chrysolampis mosquitus[a] (Linnaeus, 1758)	1 (0.6)	0.008	0	0	1	12	–	–
Amazilia lactea (Lesson, 1832)	0	0	0	1 (0.83)	0	–	–	–
Thraupidae								
Schistochlamys ruficapillus[b] (Vieillot, 1817)	3 (2.0)	0.025	–	–	3	3.3	1.5	2–5
Icteridae								
Pseudoleistes guirahuro[b] (Vieillot, 1819)	2 (1.3)	0.016	–	–	2	16.5	9.2	10–23

NV, number of visits (%); VR, visitation rate (number of visits plant^{-1} h^{-1}); IRA, Index of relative abundance (see Materials and Methods); CAP, number of individuals captured in mist nets (%), data from Costa & Rodrigues (2012); NFP, number of flowers probed per visit; N, number of visits with complete record of flowers probed.

[a] Birds observed feeding on nectar while hovering.

[b] Birds recorded feeding on nectar while perched on inflorescences.

The frequency of visitation did not reflect the relative abundance of the five hummingbirds recorded as the main flower visitors at the study site ($G = 67.6$, $df = 4$, $P < 0.001$; Table 2). The hyacinth visorbearer (*Augastes scutatus*) was the most abundant hummingbird species and corresponded to 48% of records during point counts ($n = 75$) and the most captured hummingbird at the study site (Table 2), but it was responsible for only 5% of visits to *P. robustus*. The swallow-tailed hummingbird (*Eupetomena macroura*) made up 21% of records during point counts, but only three individuals were captured (Table 2); conversely, it was the most frequent flower visitor of *P. robustus*, responsible for 60% of visits. This hummingbird species presented highly territorial behaviour, defending groups of five to seven host trees containing individuals of *P. robustus*. We recorded it chasing conspecifics and another three species that approached flowering mistletoes within its feeding territory: *E. macroura* ($n = 4$), *Augastes scutatus* ($n = 6$), *Phaethornis pretrei* ($n = 3$) and *Colibri serrirostris* ($n = 2$). The white-vented violet-ear (*C. serrirostris*) was the second most frequent flower visitor, responsible for nearly 16% of visits, but represented only 8% of records by point counts, despite being commonly captured in mist-nets (Table 2). It also presented territorial behaviour, defending flowering plants by chasing *A. scutatus* ($n = 3$), *P. pretrei* ($n = 2$) and *Chlorostilbon lucidus* ($n = 1$). The planalto hermit (*P. pretrei*) was relatively abundant, corresponding to 15% of records by point counts and being commonly captured (Table 2), but were recorded in only 6% of visits. Frequency of visitation (5%) by the glittering-bellied emerald (*Ch. lucidus*) corresponded to its relative abundance (6%) recorded during point counts, although data from mist netting indicated this species as the third most abundant species at the study site (Table 2). Another three hummingbird species were recorded as occasional flower visitors of *P. robustus* at the study site (Table 2). The cinnamon tanager (*Schistochlamys ruficapillus*) was common at the study site, usually observed in pairs, whereas the yellow-rumped marshbird (*Pseudoleistes guirahuro*) was usually observed in flocks (seven to12 birds). They fed on *P. robustus* nectar only occasionally, always perching on stem and destroying the corollas to obtain nectar; however, they also touched the stamens and stigma with their breast and belly, thus acting as potential pollinators.

DISCUSSION

Flowering strategy and reward availability

Individual plants may produce 300–600 flower buds over an extended flowering period. *P. robustus* opened flowers asynchronously, usually <10 flowers simultaneously, throughout

5 months in the rainy season, corresponding to a steady-state flowering pattern according to Rathcke & Lacey (1985). One possible advantage of this phenological strategy could be related to increased cross-pollination favouring xenogamy, since few open flowers per plant would force pollinators to move between more individuals to satisfy their energy requirements (Rathcke & Lacey 1985). Indeed, extended flowering phenology is a common pattern among other hummingbird-pollinated mistletoes (Galetto *et al.* 1990; Medel *et al.* 2002; Aizen 2003), including other species within this mistletoe genus (Buzato *et al.* 2000; Azpetia & Lara 2006; Leal *et al.* 2006; Mathiasen *et al.* 2007). The extended nectar availability related to this flowering pattern may represent resource reliability for animal visitors.

Flower anthesis lasted 3 days but nectar amount was highest in just opened flowers, when pollen is presented to pollinators. Maximum nectar accumulation (or an important amount of the total nectar produced in terms of volume) at the beginning of flower anthesis was also reported for different mistletoes, but the figure for *P. robustus* corresponds to the highest value yet reported for mistletoes (Davidar 1983; Rivera *et al.* 1996; Ladley *et al.* 1997). A single *P. robustus* flower can produce 120 μl of nectar, but the accumulated nectar produced by the flowers removed earlier reached 219 μl on average, ranging from 70 to 353 μl. In fact, the values of accumulated nectar volume recorded for *P. robustus* is three to six-fold larger than reported for other ornithophilous mistletoes (Bernhardt & Calder 1981; Ladley *et al.* 1997; Medel *et al.* 2002; Azpetia & Lara 2006; Ornelas *et al.* 2007). Ornelas *et al.* (2007) found that the amount of nectar produced in flowers was positively correlated to corolla length of ornithophilous plant species. Thus, the remarkable values of nectar volume could be related to the corolla length of *P. robustus*, which seems to have the largest flowers ever reported for a mistletoe species (Bernhardt & Calder 1981; Ladley *et al.* 1997; Medel *et al.* 2002; Azpetia & Lara 2006; Mathiasen *et al.* 2007; Ornelas *et al.* 2007).

Although flowers of *P. robustus* replenished some nectar after nectar removals, this occurred only until the midday of day 2, and refilled nectar was even more dilute. Thus, nectar removal did not represent a significant increase in the total amount of sugar secreted by flowers of *P. robustus*. Nectar sugar concentration reported for hummingbird-pollinated plants is 25% on average, but shows a wide range (Nicolson & Fleming 2003; Johnson & Nicolson 2008). Nectar sugar concentration of *P. robustus* was lower than that reported for other mistletoes species pollinated by birds (Azpetia & Lara 2006; Ornelas *et al.* 2007), ranging from 12% to 16% in those flowers sampled before the resorption phase. Production of larger volumes of nectar at lower concentrations corresponds to traits of ornithophilous species classified as pollinator-generalists (Johnson & Nicolson 2008), as observed in *P. robustus*. Conversely, the ornithophilous mistletoe *Ligaria cuneifolia* (Ruiz & Pav.) Tiegh. is pollinated by a single hummingbird species (Galetto *et al.* 1990) and produces smaller amounts of nectar at higher concentrations, on average 40 μl of nectar with a sugar concentration of 43% (Rivera *et al.* 1996). However, the amount of cumulative sugar produced per flower was similar among these two species (20 and 21 mg for *L. cuneifolia* and *P. robustus* respectively), independent of the total nectar volume (Rivera *et al.* 1996; this work).

Nectar standing crop, resorption and pollinator behaviour

Reward constancy in recently opened flowers may favour hummingbird visits and thus male reproductive success, considering early pollen presentation in mistletoes (Rivera *et al.* 1996; Aizen 2005). Female reproductive success can be also favoured during early anthesis stages, but flowers have additional chances of pollination in the next 3 days. Pollinators usually present risk-averse behaviour when they find large differences in nectar availability between flowers of the same plant (Rathcke 1992). *P. robustus* flowers produced almost all nectar in the bud stage and then, after nectar removal, many flowers did not present any reward to pollinators. Thus, if flowers receive a visit during the first day of anthesis, they do not renew a significant amount of nectar, and pollinators are faced with large variability within individual plants. This nectar pattern presentation could be related to pollinator movements to other plants when they are faced with low (or any) nectar amounts. The pattern of nectar secretion observed in *P. robustus*, combined with floral response to pollinator removals and the usual risk-averse foraging behaviour of hummingbirds, may increase pollen export and outcrossing (see below). It is expected that pollinators recognise recently opened flowers because they are the most rewarding.

The observed reduction in sugar content in bagged flowers throughout their lifetime can be inferred as active nectar resorption (Búrquez & Corbet 1991). Rivera *et al.* (1996) found that the mistletoe *L. cuneifolia* also resorbed nectar before the end of flower anthesis. The production of floral nectar implies high ecophysiological costs to plants in terms of growth and seed production (Pyke 1991). Therefore, the resorption of nectar sugars not consumed by pollinators could greatly reduce the costs of nectar production if plants are able to recover and reallocate sugars to the development of seeds and fruits (Búrquez & Corbet 1991). Nevertheless, nectar standing crop data showed that almost no further nectar was available to pollinators of the total secreted by flowers of *P. robustus* due to the high rate of hummingbird visits. This conclusion is based on floral longevity (i.e. flowers are exposed to hummingbird visits for 36 daylight hours) and the average rate of visits received by each plant (one hummingbird visit h^{-1} with *ca.* 10 flowers probed) totalling >30 visits per flower during anthesis, at least for those larger plants observed.

The role of bird pollinators in plant reproduction

We found no differences in seed set among plants pollinated with pollen from the same genet or from conspecifics, indicating that *P. robustus* is self-compatible, which seems to be a common breeding system among mistletoes (Rivera *et al.* 1996; Ladley *et al.* 1997; Robertson *et al.* 1999; Medel *et al.* 2002; Aizen 2005; Azpetia & Lara 2006). Like other mistletoes, *P. robustus* also produced seed through autonomous self-pollination but not through apomixis (Rivera *et al.* 1996; Ladley *et al.* 1997; Aizen 2005). Seed set was relatively low in autonomous self-pollinated flowers, which indicates that mistletoes could produce small amounts of seed in the absence of pollinators. However, we must consider the methodological bias of including flowers in mesh bags to determine seed set through autonomous self-pollination, because this treatment does not reflect the true outcome for unvisited flowers. In fact, the remarkable spatial gap between stigma and anthers in open flowers of *P. robustus* must limit the occurrence of autonomous self-pollination under natural conditions. Moreover, self-pollination mechanisms appear to be rare in the region considering the high rates of pollinator visits recorded for *P. robustus*. It was interesting that seed set in hand-pollinated treatments were significantly higher than in control flowers exposed to pollinators, a pattern reported for other mistletoes that indicates some degree of pollen/pollinator limitation (Medel *et al.* 2002; Aizen 2005; Robertson *et al.* 1999; Ramírez & Ornelas 2010).

The frequency of visits by *E. macroura* and *C. serrirostris* suggests that these species were the main pollinators of *P. robustus,* and have been reported as flower visitors of another ornithophilous plant species in rocky outcrops (Sazima 1977, 1981; Sazima *et al.* 1989; Vasconcelos & Lombardi 2001). These two hummingbirds were the largest species recorded and visited plants at higher frequencies than expected from their relative abundance. They usually excluded other common hummingbirds, especially the endemic *A. scutatus*, which is the most abundant nectarivorous species at the study site (Costa & Rodrigues 2012; this study). Our results indicate that dominance of flowering plants by these two hummingbirds was related to both their larger size and their defence behaviour in feeding territories that included flowering mistletoes. In addition, such behaviour indicates the value of this rich nectar source for hummingbirds during this period of the year. However, from the plant point of view, hummingbird territoriality must restrict gene flow to neighbouring plants. Indeed, pollinator behaviour observed seems to favour geitonogamy, because they usually probed many flowers per plant. Therefore, although infrequent visitors, other non-territorial hummingbirds could play a major role in promoting long-distance gene flow in this pollination system, especially *P. pretrei* known for its trapliner behaviour (Sazima 1981; Vasconcelos & Lombardi 2001). The two passerines were infrequent visitors and destroyed most of the corollas while feeding on nectar, thus their role as pollinators must be limited. Perching birds have been reported as flower visitors of other mistletoes, but their role as pollinators remains controversial (Graves 1982; Rocca & Sazima 2008).

The local guild of birds feeding on nectar of *P. robustus* is one of the largest reported for a mistletoe species. Gill & Wolf (1975) observed seven species of sunbird (Nectariniidae) visiting flowers of *Phragmanthera dshallensis* (Engl.) Balle, and these birds also actively defended their nectar source. Other studies have reported up to five species of nectarivorous bird visiting flowers of mistletoes (Bernhardt & Calder 1981; Ladley *et al.* 1997). Some South American mistletoe species have restricted pollination systems that depend on a single hummingbird species for pollen delivery (Galetto *et al.* 1990; Medel *et al.* 2002; Aizen 2003). Even in tropical communities, *Psittacanthus* spp. have up to four bird species as flower visitors (Feinsinger 1978; Buzato *et al.* 2000; Araújo & Sazima 2003; Azpetia & Lara 2006; Leal *et al.* 2006; Rocca & Sazima 2008). In addition, the local guild of nectarivorous birds feeding on *P. robustus* is also larger than reported for other ornithophilous plant species elsewhere in mountain rocky outcrops in the Espinhaço Range (Vasconcelos & Lombardi 2001; Machado *et al.* 2007).

Our data are in accordance with Watson (2001), who considered mistletoes as potential keystone resource for vertebrates. *P. robustus* is typically an ornithophilous plant that produces many flowers with high quantities of nectar and sugar rewards over an extended period throughout the year. Individual mistletoes bearing up to 400 flowers may produce over 10 g nectar sugars, more than the total weight of the heaviest hummingbird species observed as visitor. It is important to highlight that *P. robustus* is not redundant with its host trees in terms of resource provided to fauna of highland rocky savannas. Melastomataceae hosts offer only pollen as reward and are pollinated by large bees, whereas Vochysiaceae are mostly pollinated by bees and moths, with hummingbirds as opportunist flower visitors (Gottsberger & Silberbauer-Gottsberger 2006). The wide use by birds indicates that *P. robustus* may represent a keystone resource, and this species could be positively related to local diversity of nectarivorous species in the Serra do Cipó Mountains.

Linking nectar secretion to pollinator behaviour and plant reproductive success

Flowers produced a high amount of nectar in the bud stage and replaced a small amount after a visit (i.e. experimental nectar removal). Nectar standing crop showed that flowers usually offered a small amount of nectar, and many flowers did not have any nectar for pollinators. Nectar standing crop is related to the nectar production/resorption rate and the frequency of pollinator visits. The recorded low standing crop in flowers of *P. robustus* it is not a surprise because each flower renewed a little nectar after a removal and received a high number of visits during the day. Thus, nectar resorption seems to be a rare event in this context, except for those days of bad weather conditions that would represent an important energy-saving mechanism for uncollected nectar. In fact, the capacity of nectar resorption seems to be clear, indicative of high energetic costs related to nectar secretion for this mistletoe.

Flowers exposed to hummingbirds presented a flower/seed ratio significantly lower than hand-pollinated flowers. Indeed, hummingbirds usually probed many open flowers of an individual that were at different age stages; a single flower might receive >30 visits during its lifetime. Therefore, reproductive constraints must not be related to flower visitor scarcity but to their effectiveness as pollinators and trade-offs among energy allocated to produce nectar and seeds. According to Ornelas & Lara (2009), the optimum number of visits promotes deposition of intermediate pollen loads, which favour higher female plant reproductive success. For example, many flowers of a plant cannot be pollinated or receive poor pollen loads if the number of visits is very low, leading to a low seed set. Likewise, if the number of visits is too high, stigmas can be damaged or pollen removed due to the excessive number of visits, also leading to a low seed set. The spatial gap among anthers of *P. robustus* flowers favoured pollen deposition on flapping wings of hovering hummingbirds; therefore, this could be one explanation for the lower seed set recorded for naturally pollinated flowers in comparison to hand-pollinated flowers, despite the high rates of hummingbird visits observed during the flowering season. Moreover, this limitation to seed production could also be related to a trade-off between nectar secretion and seed production (Ordano & Ornelas 2005; Ornelas & Lara 2009). Data on nectar standing crop and flower visitation indicated that hummingbird pollinators removed most of the secreted nectar throughout flower anthesis; as consequence, there could be less energy available for flowers to invest in seed production, whereas the absence of nectar removal in hand-pollinated flowers led to the sugar rewards being reabsorbed, thus explaining the larger seed set observed.

Psittacanthus robustus seems to be a keystone nectar resource for birds, whereas flower visitation by hummingbird pollinators is a critical event for mistletoe reproduction. Conversely, the high flower visitations by territorial hummingbirds seem to be excessive for the plant, considering the female reproductive success reached by naturally pollinated flowers. Therefore, we conclude that the costs and benefits of mistletoe reproduction depend on the interaction strength between flowers and their pollinators. Assessment of nectar secretion dynamics, pollinator behaviour and plant breeding system allowed elucidation of the complexity of these associations.

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