Reproductive biology and nectar production of the Mexican endemic *Psittacanthus auriculatus* (Loranthaceae), a hummingbird-pollinated mistletoe

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**Keywords**
- Floral biology; hummingbird pollination; Loranthaceae; pollination syndromes; Psittacanthus; Trochilidae.

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**ABSTRACT**

Many mistletoe species produce ‘bird’-pollinated flowers; however, the reproductive biology of the majority of these species has not been studied. *Psittacanthus auriculatus* is a Mexican endemic mistletoe, most common in open, dry mesquite grassland. Knowledge of the reproductive biology of *P. auriculatus* is essential for understanding species formation and diversification of *Psittacanthus* mistletoes, but it is currently poorly understood. Thus, we studied floral biology and phenology, nectar production and breeding system and pollination of this species. The hermaphrodite red-pink flowers open from the middle to the tip and petals are curly, but remain partially fused forming a floral tube of ca. 20-mm long. Flowers are partially protandrous, produce large amounts of nectar, last 2 days, and stigma receptivity is highest during the second day. We recorded hummingbirds (*Cynanthus latirostris*, *Hylocharis leucotis*, *Amazilia beryllina*, *A. violiceps*, *Calothorax lucifer*, *Archilochus colubris*) and less commonly butterflies (*Aagraulis vanillae*, *Anteos clorinde*, *Papilio multicaudatus*, *Phocides urania*, *Phoebis sennae*) as floral visitors. *P. auriculatus* flowers are self-compatible. However, this mistletoe is an obligate animal-pollinated species, as the sensitive stigma avoids self-pollination. Under natural conditions, reproductive success was higher than in manually selfed or cross-pollinated flowers, likely due to the trapping foraging behaviour of hummingbirds. We suggest that the apparent efficient foraging behaviour of hummingbirds maintains gene flow among *P. auriculatus*, promoting outcrossing.

**INTRODUCTION**

The exceptional species richness and increased diversification rates of angiosperms have frequently been associated with a number of floral features for biotic pollination (Stebbins 1970; Vamosi & Vamosi 2010). The functional significance of floral traits and the plant reproductive consequences of the foraging behaviour of animal pollinators have been a traditional subject of pollination biology. Recently, a plethora of studies of modified flowering systems, floral traits responsible for the maintenance of the specific interaction (e.g. flower size, amount of floral reward) are expected to be strongly correlated with the pollinator morphology and its behaviour for floral reward collection, enhancing the syndromes.

Loranthaceae is the largest and most diverse family (73 genera and ca. 990 species) distributed in the Old and New World tropics (Vidal-Russell & Nickrent 2008a; Nickrent et al. 2010). Most Loranthaceae species are dependent exclusively on a bird agent for pollination and dispersal, whereas entomophily and autonomous pollination are less frequent...
Acacia (Kuijt 1969; Bernhardt & Knox 1983; Mathiasen et al. 2008). The massive adaptive radiation of Loranthaceae is linked to the presence of stem parasitism (Vidal-Russell & Nickrent 2008a,b), which was probably fuelled by their interactions with pollinating birds (Vidal-Russell & Nickrent 2008a). In the Old World, the evolutionary history of Loranthaceae was linked to the interaction with Meliphagidae (honeyeaters) in New Zealand and Australia (Barker et al. 2004), in Asia and Africa with Nectariniidae (sunbirds) and Dicaeidae (flowerpeckers; Docters van Leeuwen 1954; Gill & Wolf 1975; Barker et al. 2004; Beresford et al. 2005), and in the New World, with Trochilidae (hummingbirds; Vidal-Russell & Nickrent 2008a). Many tropical and subtropical Loranthaceae mistletoes have large, colourful flowers borne in groups that produce large amounts of sugar-rich nectar that attract avian pollinators. Elaborate pollination mechanisms involving birds have evolved in some of the Old World Loranthaceae species, in which birds pry open the fused corollas to reach their nectar reward, upon which the pollen ‘explodes’ onto the bird’s head (Ladley et al. 1997). These mistletoes are often dichogamous (protandrous), and after the birds have visited flowers in the male phase, they eventually visit flowers in the female phase, thereby effecting pollination (Mathiasen et al. 2008). However, a broad range of species including hummingbirds and insects service most New World Loranthaceae mistletoes, and no nectarivorous pollinator is yet considered as a mistletoe specialist (Watson 2001).

Psittacanthus Mart. (ca. 120 species) is the largest genus within Loranthaceae distributed from Mexico to northern Argentina (Kuijt 2009, 2014), with a minor representation in the Caribbean Islands (Kuijt 2009). The green or yellow to orange or brilliant red flowers of Psittacanthus are one of the most striking features of these hemiparasitic plants, and no other genera of Loranthaceae has its structural variability probably linked to pollination. Flowers are hermaphroditic, generally actinomorphic or slightly zygomorphic with strongly curved flower buds, and a large variation in flower lifespan, flower color changes through time, apparent petal fusion, diameter of the floral tube and anthers, and in the petal curliness (Kuijt 2009). Despite this, data on their pollination ecology have been published for only three species: P. calycatus (DC.) G. Don (Aspeltia & Lara 2006), P. schiedeanus (Schltdl. & Cham.) Blume (Ramírez & Ornelas 2010) and P. robustus (Mart.) Mart. (Guerra et al. 2014). In these studies, the breeding system and nectar production patterns are described to assess the interaction between the mistletoe and its floral visitors. P. auriculatus (Oliv.) Eichler is a narrow endemic to the Mexican states of Oaxaca and Puebla, between 1300–2000 m a.s.l. (Kuijt 2009). It is the only Mesoamerican species with cordate leaves parasitising mainly Acacia and Prosopis trees (Kuijt 2009). Even though its flowers resemble phenotypic specialisation to hummingbird pollination, no studies on its reproductive biology exist. Here we studied the pollination biology of P. auriculatus and hypothesised that it has phenotypic and functional specialisation to hummingbirds. Specifically, we describe the floral biology, nectar secretion dynamics, floral visitors, breeding system and pollination of P. auriculatus inhabiting thorn forests of Oaxaca, Mexico.

MATERIAL AND METHODS

Study area

The study was conducted in the period of maximum flowering of the species (August–September) during the flowering seasons of 2013 and 2014 at Predio Fandango in the vicinity of Santiago Matatlán, Oaxaca, Mexico (16°52‘N; 96°23‘W; 1740 m a.s.l.). Santiago Matatlán is situated in the southeast portion of the Valles Centrales, a region composed of three pluvial valleys (the Etla valley in the northeast, Tlacolula towards east, and Zimatlan-Ocotlan valley to the south) and surrounded by the Nudo Mixteco, Sierra de Juárez and Sierra Madre del Sur mountain ranges. The study area south of Oaxaca City is a dry region characterised by a summer rainy season (May–October) and mean annual precipitation of 635.25 mm, with a minimum of 208 mm in December and a maximum of 1198 mm in July (Pérez-Barroso 2009; Instituto Nacional para el Federalismo y Desarrollo Municipal, http://www.inafed.gob.mx/work/enciclopedia/EMM200oaxaca/index.html). The mean annual temperature is 20°C, with a range of 8–12°C minimum in December and January and a maximum range of 28–35°C from March to May (Pérez-Barroso 2009). The vegetation comprises an association of induced grassland combined with Prosopis laevigata and Acacia farnesiana (thorn forest, mesquite grassland or bosque espinoso; Rzedowski 2006).

Floral biology

In August 2013, we selected ten mistletoes growing on 10 Acacia farnesiana Wall. (Fabaceae) host trees and measured 30 floral buds and 30 flowers from each of the plants (n = 300 buds, n = 300 flowers). For fully developed buds and flowers we measured pedicel and corolla tube length, and for flowers we measured the lengths of the pistil, filament and anthers (error: 0.05 mm).

To evaluate floral longevity, we conducted daily inspections following 60 tagged buds in 19 mistletoes growing on 18 different host trees until wilting during August 2013. We also recorded stigma receptivity by submerging stigmas into a 32% hydrogen peroxide solution and using the presence of bubbling on the stigma to infer receptivity (Kearns & Inouye 1993). Stigma receptivity of 91 flowers was recorded over 5 days after flower opening (19 on day 1, 18 on day 2, 25 on day 3, 14 on day 4, 15 on day 5).

Floral visitors

In August 2013, we randomly selected 36 individual mistletoes on host trees in the study area and surroundings to determine the identity and foraging patterns of the floral visitor species. We performed 12.3 h of focal observations (20 min-plant⁻¹) at different periods of the day, beginning at 07:30 h until 16:40 h on different days. In August 2014, we performed an additional 20 h of focal observations on 60 additional plants, as described above. We used binoculars (12 × 50, Eagle Optic) and digital photo and video cameras (Sony Alpha 300–500 mm, Sony Handycam HDR-CX190 5.3 megapixels, New York, USA) to record the visitors. Birds and insects were identified using photos and videos with the aid of field guides (Williamson 2001; Glassberg 2007). We identified pollinators according to their

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behaviour, and whether they made contact with both anthers and stigma.

Breeding system

To evaluate the breeding system of *P. auriculatus*, we conducted a manual-pollination experiment using flowers of 15 mistletoe plants growing on 15 *A. farnesiana* host trees and bagged buds about to open in mesh bags (1-mm bridal tulle) to exclude floral visitors in August 2013. Six pollination treatments (autonomous self-pollination, autogamous hand-self, hand-geitonogamy, hand-outcross, open pollination, apomixis) were applied to each plant (>50 flowers per treatment). Self- and cross-pollination treatments were applied 24 h after flower opening (day 2). The manual autogamy treatment was carried on day 1, as pollen grains from donor flowers remain just a few hours in the anthers. To test for autonomous self-pollination and fruit set, flowers about to open were bagged to exclude floral visitors (Treatment 1: autogamy, n = 51 flowers). In the hand-self treatment, flowers were pollinated by brushing their own anthers onto the stigma; flowers were previously emasculated by cutting off the stamens to prevent further contact between pollen and stigma, and floral visitors were excluded by enclosing the inflorescence in mesh bags until fruit maturation (Treatment 2: manual autogamy, n = 54). To test for self-compatibility in the hand-geitonogamy treatment, we emasculated flowers on their opening day and hand-pollinated flowers (day 2) by brushing anthers from another flower of the same individual plant and excluded pollinators by enclosing the flowering branch as explained above (Treatment 3: geitonogamy, n = 75). In the hand-outcross treatment, flowers were emasculated immediately after opening and pollinated by smearing one anther from an arbitrarily selected pollen donor onto the receptive virgin stigma, and pollinators excluded by enclosing the flowering branch as explained (Treatment 4: xenogamy, n = 56). We assessed natural pollination and fruit set from a different group of flowers that remained unbagged and open to natural pollination (Treatment 5: open pollination, n = 58). This treatment acted as a control for seed production, although the mechanisms affecting seed production in this treatment are unclear and may be due to resource allocation, pollen loads or both, as shown in other Loranthaceae species (Robertson et al. 1999; Montgomery et al. 2003). Finally, a different group of flowers about to open were emasculated and bagged to test for apomixis (Treatment 6: apomixis, n = 54). Fruits from experimental flowers were collected and quantified 2 months later.

In September 2014, the contribution of autogamy to pollination success was further evaluated on individuals growing on ten *A. farnesiana* host trees, the same host plants used to test the breeding system in August 2013. Two treatments were applied: flowers emasculated on day 1 and then left open to natural pollination (Treatment 7: emasculated open pollination, n = 59), and intact flowers remained open at the same time (Treatment 8: control, n = 62). Fruits of these treatments were also counted 2 months later.

Nectar secretion dynamics

Nectar production, effects of nectar removal on total production and standing crops were evaluated to determine reward availability for pollinators over time in August 2013. Nectar was extracted from flowers using calibrated micropipettes (10 μl) and quantified with the aid of a digital Vernier (error: 0.1 mm). Sugar concentration (percentage sucrose) was measured with a pocket refractometer (VEEGEE BTX-1, range of concentration 0°–32° Brix scale). Sugar amount produced was expressed as milligrams sugar per ml solution, after Kearns & Inouye (1993). First, we quantified the amount of nectar accumulated throughout the life of individual flowers at 24-h intervals. Buds ready to open of selected inflorescences from mistletoes growing on different host trees (N = 15) were bagged and excluded from floral visitors to allow nectar to accumulate. The accumulated nectar was extracted on the following day after exclusion at 08:00 h (day 1, n = 68) and then flowers were removed from the plant. The same procedure was repeated on a different group of flowers in which the nectar was allowed to accumulate and removed 24 h (day 2, n = 70) and 48 h (day 3, n = 77) after exclusion. Second, total nectar production with repeated removals was evaluated on a different group of mistletoe flowers from 11 different mistletoe individuals growing on different host trees of *A. farnesiana* (N = 10). Buds ready to open were bagged to exclude floral visitors, and once the flower opened the nectar produced in a day was measured at 3-h intervals (08:00, 11:00, 14:00 and 17:00 h) throughout the life of the flower (3 days) to explore the capacity of *P. auriculatus* flowers to replenish the repeatedly removed nectar. Flowers (n = 51) were not removed from the plant after the extraction of nectar and were kept bagged between removals of nectar. Nectar was carefully removed, avoiding nectar damage. On day 3, flowers began to drop their petals and nectar could only be removed at 08:00 h, and no nectar was found at later time intervals. Finally, because pollinators probably respond to nectar standing crop, we extracted every 2 h the available nectar in 1034 flowers (n = 67 at 08:00, n = 70 at 10:00, n = 198 at 12:00, n = 334 at 14:00, n = 276 at 16:00, and n = 89 at 18:00 h) from 26 mistletoe individuals growing on six host trees that were exposed to floral visitors, and measured its volume and concentration to evaluate variation in nectar standing crops during the period of pollinator activity.

Data analysis

Floral visitation data were standardised as number of visits per plant in 20 min of focal visualisation by period of time. Period of observation time was set to 2 h (08:00 include all records between 08:00 and 10:00, etc.). Observed visitation frequency by species and functional group was compared with the goodness-of-fit G test (Zar 1999). Statistical significance of each treatment (species and functional group) between observed and expected frequencies was calculated with Haberman’s (1973) standardised residuals. Differences in fruit production between pollination treatments were assessed using a chi-square test (Zar 1999), and the Haberman’s (1973) standardised residuals were calculated to establish the statistical significance of each treatment. To assess the contribution of autonomous self-pollination to fruit production (pollination experiment 2014), a generalised linear model (GLM) in RStudio version 0.98.490 (R Development Core Team; http://www.r-project.org/) was performed with binomial error and a logit link function. The full GLM model
included pollination treatment treated as fixed effects and fruit production as a binary response variable. Plant identity was included in a second model as random effects, and the model with the lowest Akaiki information criterion (AIC) was selected as the best model (Akaiki 1981). Lastly, two indices were calculated to describe the breeding system: (i) self-incompatibility system (ISI), calculated by dividing the percentage of fruit set with xenogamy. Self-compatible species score ≥1, incompletely compatible species score values between 1 and 0, and self-incompatible species score 0. (ii) Automatic self-pollination (IAS) was calculated as the ratio between the natural autogamous fruit set and the manually autogamous, actinomorphic, chorypetal and petals are 37.9/1.20 mm, width 3.8/3.47-mm long. There are usually six stamens (nectar volume and amount of sugar) as continuous response variables. Data of nectar production (volume and amount of sugar) after removing nectar every 3 h and for 3 days were analysed using a generalised additive mixed model (GAMM) in R including number of removals (from 1 to 12) treated as fixed effects and nectar production as the repeated measures (Lin & Zhang 1999). The mixed effects and GAMM models were also run adding the random effects of plant identity, and the model with the lowest AIC was selected as the best model (Akaiki 1981). To assess the effects of nectar removal, a linear mixed effects model was performed on total nectar production, comparing flowers from which nectar was repeatedly removed (five removals up to 08:00 h, day 2) and flowers from which nectar was allowed to accumulate until day 2 (removed at 08:00 h, day 2). Simple contrasts were performed to compare means between pairs in all models (Pinheiro & Bates 2000). Lastly, a Kruskall-Wallis test was used to compare standing crop nectar removal between hours. Differences between groups were evaluated with Games–Howell’s (1976) multiple comparisons (Zar 1999). These statistical analyses were performed using SPSS Statistics version 21 (IBM Corp., Armonk, NY, USA) and StatView version 5.0 (SAS Institute, Cary, NC, USA).

RESULTS

Floral biology

Inflorescences of *P. auriculatus* are terminal and axillar, and buds are exposed in triads (Fig. 1A). Buds present a slight swelling in the distal portion prior to flower opening. Flowers are hermaphrodite and the calyx is inferior (mean ± SE, length 4.0 ± 1.20 mm, width 3.8 ± 0.63 mm). The corolla is hexamorous, actinomorphic, chorypetal and petals are 37.9 ± 3.38-mm long (Table 1). Flowers open from the middle to the tip and petals curly but remain partially fused forming a floral tube 19.6 ± 3.47-mm long. There are usually six stamens (32.5 ± 3.42-mm long) as many as petals, and red-pink in colour. The bright yellow pollen in anthers (5.9 ± 1.16-mm long) contrast to the red pink-red floral tube and red filaments (Fig. 1B). The red style (38.2 ± 3.15 mm) is longer than the filaments (32.5 ± 3.52 mm; Table 1), which hold the anthers near the style, and the stigma is papillate. Stigma receptivity varied significantly with flower age (G = 41.33, df = 4, P < 0.001), increasing from 10.5% during flower opening up to 83% on the second day (P < 0.01). On day 3, stigma receptivity decreased to 32%. Petals last attached to the flower for about 2 days (2.4 ± 0.09 days), and usually fall by the third day (48 h after opening). Corolla colour changes with age from red-pink on the first day or two to pink orange with senescence. Anthers are dorsifixed, anther dehiscence in all tagged flowers started as they open, and pollen grains remained attached to thecae for a few hours (even in bagged flowers). However, most pollen is removed within the first day in anthers exposed to pollinators (Fig. 1C).

*Psittacanthus auriculatus* exhibited a partially protandrous breeding system. Flowers on their first day of anthesis developed a ca. 12 h male phase (staminate), followed by some overlap of a few hours with the ca. 36 h female phase (pistillate phase). At the beginning of anthesis, the style is shorter than the stamens and projects far above the stamens; the stigma stands erect whereas the filaments become shorter and positioned with a remarkable spatial gap between stigma and anthers (Fig. 1D). Because growing stigmas make physical contact with the anthers, this very short transitional stage can be delineated as the zone at which autonomous self-pollination can theoretically take place.

Floral visitors

We recorded 174 visits from six hummingbird species in a period of 32 h of focal observations. Hummingbirds (*Cynanthus latirostris*, *Hylocharis leucotis*, *Amazilia beryllina*, *A. violiceps*, *Archilochus colubris*, *Calothorax lucifer*) were the main group of visitors of *P. auriculatus*, accounting for 34% of all visits. Butterflies (*Agraulis vanillae*, *Anteos clorinde*, *Papilio multicaudatus*, *Phoebis urania* and *Phoebea sennae*) were occasionally recorded (8% of all recorded visits). *Apis mellifera* accounted for 58% of all recorded visits. Honeybees were most frequent during the morning hours (Fig. 2), collecting pollen voraciously and some individuals robbing nectar through the fenes- trae (Fig. 1D). When visiting flowers in the male phase (low stigma receptivity), honeybees collected pollen grains by grooming into scopae. These pollen grains are probably wasted for deposition on a receptive stigma. During the female phase (2-day flowers), the stigma was not contacted. Hummingbirds were the main floral visitors and were active throughout the day (Fig. 2), probing few flowers per visit. When landing on the inflorescence or hovering beside the flower to take nectar (Fig. 1F), hummingbirds made no contact with the anthers or stigma. More often, however, during hovering visits over the flowers (Fig. 1G–H), anthers and stigma were usually contacted. *Bomhus* species were occasionally observed visiting *P. auriculatus* flowers, but due to their mismatch and nectar foraging behaviour on flowers they did not contact anthers and stigma. Some small and large butterflies (Fig. 11), and less frequently wasps, also visited *P. auriculatus* flowers to collect or rob nectar, but anthers and stigma were not contacted. The beyrilline hummingbird (*Amazilia beryllina*) was the most frequent flower visitor of *P. auriculatus*, responsible for 54% of
174 hummingbird visits. The violet-crowned hummingbird (Amazilia violiceps) was the second most frequent flower visitor, responsible for nearly 24% of visits, and the ruby-throated hummingbird (Archilochus colubris) was the third most frequent flower visitor (17% of all visits). The lucifer hummingbird (Calothorax lucifer), broad-billed hummingbird (Cynanthus latirostris) and white-eared hummingbird (Hylocharis leucotis) were occasional floral visitors of P. auriculatus at the study site, with <2% of all visits for each species. None of the hummingbird species displayed territorial behaviours during our observations.

Breeding system

Low female reproductive success was observed after hand-self- and cross-pollination treatments (Table 2). However, flowers exposed to natural pollination produced significantly more fruits than those exposed to either pollination treatment (Table 2). Accordingly, fruit set was not independent of pollination treatment ($\chi^2 = 51.4$, df = 5, $P < 0.0001$), particularly in flowers open to natural pollination in which fruit set was higher than expected (60.5%, $P < 0.01$) and, in apomixis, lower than expected (9.3%, $P < 0.01$). No significant differences in fruit set were found between autonomous self-pollination (21.5%), manual self-pollination (25.4%), self-compatibility (21.3%) and cross-compatibility (20.7%). There were no significant differences in fruit production between emasculated and non-emasculated (control) flowers open to natural pollination (pollination treatment effects: mean ± SE, estimate = 0.05 ± 0.38, z value = 0.13, $P = 0.89$; AIC = 162.41 for the model with plant identity as random effects, AIC = 162.17 for the model without plant identity as random effects, $P = 0.184$). This means that the contribution of autogamy to natural pollination is minor.

According to the self-compatibility index (ISI = 1.03), P. auriculatus is self-compatible, with a similar female reproductive success between hand-self and hand-cross pollination. Automatic self-pollination index (IAS) equals 0.84, which means that flowers are partially autogamous, and a higher autonomous reproductive success is achieved by hand-pollinated flowers than those after autonomous pollination.

Fig. 1. Psittacanthus auriculatus morphology and floral visitors in a mesquite grassland at Santiago Matatlán, Oaxaca, Mexico. A: Inflorescence and flower morphology. Note the open flower with recently dehiscent anthers and yellow pollen (staminate). B: Unvisited 1-day flower (staminate). Note the prominent style (arrow) among the stamens. C: One-day flower with empty anthers (arrow) after pollen collection by honeybees (Apis mellifera). D, E: Apis mellifera robbing nectar from a 2-day flower (pistillate). F: Amazilia violiceps perched on the inflorescence while accessing nectar. G: Amazilia violiceps hovering and taking nectar during a visit. H: Archilochus colubris hovering during a visit. Note that when hummingbirds visit hovering over a P. auriculatus flower the anthers and stigma make contact with their foreheads or throats. I: Papilio multicaudatus resting on a staminate flower while accessing nectar.
Table 1. Flower and bud measurements (mm) for Psittacanthus auriculatus (Loranthaceae) in a mesquite grassland, central Oaxaca, Mexico.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>n</th>
<th>range</th>
<th>minimum</th>
<th>maximum</th>
<th>mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>flowers</td>
<td>10</td>
<td>310</td>
<td></td>
<td>16.50</td>
<td>3.90</td>
<td>20.40</td>
</tr>
<tr>
<td>Floral tube length</td>
<td>29.20</td>
<td>11.20</td>
<td>40.40</td>
<td>19.60 ± 3.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Style length</td>
<td>28.30</td>
<td>18.40</td>
<td>46.70</td>
<td>38.20 ± 3.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filament length</td>
<td>28.90</td>
<td>10.60</td>
<td>39.50</td>
<td>32.47 ± 3.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filament lengthb</td>
<td>15.20</td>
<td>5.50</td>
<td>20.70</td>
<td>13.76 ± 2.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anther length</td>
<td>5.70</td>
<td>3.10</td>
<td>8.80</td>
<td>5.94 ± 1.16</td>
<td></td>
<td></td>
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<tr>
<td>Ovary length</td>
<td>6.00</td>
<td>2.00</td>
<td>8.00</td>
<td>3.97 ± 1.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary width</td>
<td>4.30</td>
<td>1.70</td>
<td>6.00</td>
<td>3.83 ± 0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of petals</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>6 ± 0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of stamens</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>6 ± 0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pedicel length</td>
<td>12.00</td>
<td>8.30</td>
<td>20.30</td>
<td>13.77 ± 2.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Buds                           | 10 | 310 |       | 19.80   | 25.60   | 45.40     | 37.89 ± 3.38 |
| Bud length                     | 19.80 | 25.60 | 45.40 | 37.89 ± 3.38 |
| Ovary length                   | 3.40 | 1.70 | 5.10  | 3.15 ± 0.53 |
| Ovary width                    | 2.10 | 2.90 | 5.00  | 3.90 ± 0.38 |
| Pedicel length                 | 12.00 | 8.30 | 20.30 | 13.77 ± 2.16 |

N, mistletoe individuals; n, flowers.

*a*Measurement taken from attachment to petal to the tip.

*b*Measurement taken from attachment to petal to the tip.

Fig. 2. Visitation frequency of potential groups of pollinators of *Psittacanthus auriculatus* flowers in a mesquite grassland, central Oaxaca, Mexico. Visitation frequency data (%) are given for 20-min observation periods throughout the day.

Table 2. Fruit set (number of fruit/number of flowers) of *Psittacanthus auriculatus* by pollination treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>n</th>
<th>fruits</th>
<th>fruit set %</th>
<th>residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>autonomous self-pollination (autogamy)</td>
<td>12</td>
<td>51</td>
<td>11</td>
<td>21.57</td>
<td>−0.84</td>
</tr>
<tr>
<td>manual self-pollination (manual autogamy)</td>
<td>15</td>
<td>55</td>
<td>14</td>
<td>25.45</td>
<td>−0.32</td>
</tr>
<tr>
<td>self-compatibility (geitonogamy)</td>
<td>14</td>
<td>75</td>
<td>16</td>
<td>21.33</td>
<td>−1.50</td>
</tr>
<tr>
<td>cross-compatibility (xenogamy)</td>
<td>13</td>
<td>58</td>
<td>12</td>
<td>20.69</td>
<td>−1.02</td>
</tr>
<tr>
<td>apomixis</td>
<td>13</td>
<td>54</td>
<td>5</td>
<td>9.26</td>
<td>−2.58**</td>
</tr>
<tr>
<td>open to pollinators (control)</td>
<td>15</td>
<td>71</td>
<td>43</td>
<td>60.56</td>
<td>5.25**</td>
</tr>
</tbody>
</table>

N, plants; n, flowers.

**P < 0.01.

Nectar secretion dynamics

*Psittacanthus auriculatus* flowers produced copious dilute nectar. Data presented in Table 3 describe the natural secretion dynamics of flowers excluded from floral visitors throughout anthesis. Nectar was present at the time of flower opening and from the beginning of anthesis flowers produced nectar for 2 days and stopped producing nectar on day 3. Nectar accumulation varied significantly among flowers of *P. auriculatus* (flower age effects: nectar volume, $F_{2,197} = 33.84, P < 0.0001$; amount of sugar, $F_{2,197} = 30.93, P < 0.0001$; Fig. 3). The mixed effects model including plant identity as random effects better fit the data for both volume ($AIC = 1686.04$ for the model with plant identity as random effects, $AIC = 1700.28$ for the model without plant identity as random effects, $P < 0.0001$) and amount of sugar ($AIC = 1243.13$ for the model with plant identity as random effects, $AIC = 1250.89$ for the model without plant identity as random effects, $P = 0.0018$). The nectar volume per flower increased from 25.2 µl on day 1 to 31.2 µl on day 2, and then decreased to 13.6 µl after 48 h of accumulation (day 3). A similar tendency was observed for the amount of sugar per flower (mg sugars), with an increase from 7.9 mg on day 1 to 10.8 mg on day 2, and a decrease to 4.8 mg on day 3. In summary, the amount of nectar is higher during the pistillate phase of the flowers (day 2).

Experimental effects of repeated removal (nectar repeatedly removed one to 12 times) on total nectar production showed that the amount of nectar varied significantly among removals (removal intensity effects: nectar volume, $F_{12,550} = 62.87, P < 0.0001$; amount of sugar, $F_{12,550} = 50.11, P < 0.0001$; Fig. S1). The GAMM models excluding plant identity as random effects better fit the data for both volume ($AIC = 3748.16$ for the model with plant identity as random effects or without it) and amount of sugar ($AIC = 2073.27$ for the model with plant identity as random effects or without it). The amount of nectar removed at 08:00 h on day 1 and day 2 almost accounted for the total amount of nectar secreted per flower during anthesis. Furthermore, the amount of nectar removed on the first removal of day 1 was higher than the amount removed on the fifth removal on day 2 for volume ($15.7 versus 12.2 µl, P < 0.001$) but not for the amount of sugar (3.0 versus 3.3 mg, $P > 0.05$), suggesting that sugar production is not reduced with repeated removal. The set of removals 9 to 12 (day 3) did not account for total production of nectar, and no
Table 3. Nectar production pattern throughout flower anthesis of Psittacanthus auriculatus mistletoes growing on Acacia farnesiana in a mesquite grassland, central Oaxaca, Mexico.

<table>
<thead>
<tr>
<th>R</th>
<th>TAO</th>
<th>TOR</th>
<th>nectar treatment</th>
<th>accumulated</th>
<th>removed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>nectar (µl)</td>
<td>sugar (mg)</td>
<td>°brix</td>
</tr>
<tr>
<td>day 1</td>
<td>1</td>
<td>0</td>
<td>8:00</td>
<td>25.20 ± 10.70</td>
<td>7.93 ± 3.73</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>11:00</td>
<td>2.96 ± 3.87</td>
<td>0.78 ± 1.12</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6</td>
<td>14:00</td>
<td>2.73 ± 4.17</td>
<td>0.85 ± 1.35</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td>17:00</td>
<td>1.37 ± 3.40</td>
<td>0.46 ± 1.24</td>
</tr>
<tr>
<td>day 2</td>
<td>5</td>
<td>24</td>
<td>8:00</td>
<td>31.23 ± 13.92</td>
<td>10.77 ± 4.53</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>27</td>
<td>11:00</td>
<td>0.40 ± 1.46</td>
<td>0.10 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>30</td>
<td>14:00</td>
<td>0.10 ± 0.52</td>
<td>0.03 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>33</td>
<td>17:00</td>
<td>0.05 ± 0.21</td>
<td>0.01 ± 0.06</td>
</tr>
<tr>
<td>day 3</td>
<td>9</td>
<td>48</td>
<td>8:00</td>
<td>13.60 ± 13.82</td>
<td>4.85 ± 4.97</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>51</td>
<td>11:00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>54</td>
<td>14:00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>57</td>
<td>17:00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

R, number of removals; TAO, time elapsed from flower opening in hours; TOR, time of removal.

Data are means ± SD.

Fig. 3. Nectar accumulated of 3-day P. auriculatus (Loranthaceae) flowers from 15 plants. A: Nectar volume. B: Amount of sugar. Mean ± SE.

differences were found between removals (P > 0.05). Thus, removals 1 to 5 were used to evaluate the removal effects on cumulative nectar production. The sum of nectar secreted by flowers after repeatedly removing the nectar five times was added and compared with the amount of nectar accumulated for 24 h. No significant effect of repeated removal was found on the volume of nectar secreted (nectar removal effects: F_{1,105} = 1.28, P > 0.05; Fig. S2) but significantly reduced the amount of sugar (F_{1,105} = 6.79, P < 0.05). Amount of nectar accumulated on day 1 (male phase) is 80% of the total volume and 73% of the total sugar secreted.

Nectar standing crop per flower was very low throughout the day in terms of volume or amount of sugar, but differed significantly over time (volume, n = 1034, H = 53.53, df = 5, P < 0.001; amount of sugar, n = 847, H = 61.92, df = 5, P < 0.001; Fig. 4). Higher nectar standing crops were observed during the morning collections at 08:00 and 10:00 h (mean ± SD: volume 1.81 ± 3.6 µl; amount of sugar 0.50 ± 1.01 mg), and decreased by noon (volume 0.13 ± 0.27 µl; amount of sugar 0.02 ± 0.09 mg). The percentage of flowers with standing nectar varied throughout the day, with 27% of all measured flowers contained some nectar at 10:00 h, 4% at 12:00 h, and 32% of all flowers that contained some nectar were measured in the afternoon (14:00 h, 16:00 h, 18:00 h).

DISCUSSION

Breeding system

The morphological floral features of P. auriculatus fit the syndrome of ornithophily (Faegri & Van der Pijl 1979) and appear to be adapted for hummingbird pollination, as previously suggested for some Psittacanthus species (P. laterifolius, Feinsinger 1978; P. dichrous, Buzzato et al. 2000; P. calyculatus, Azpeitia & Lara 2006; P. cordatus (=byclayculatus), Leal et al. 2006; P. schiedeanus, Ramírez & Ornelas 2010; P. robustus, Guerra et al. 2014). Flowers of P. auriculatus are long (38 mm), bright red-pink in colour, petals form a floral tube which remains tubular with age, filaments and style are erected favouring pollination by hummingbirds while removing nectar from the top (Castellanos et al. 2003). The flowers last ca. 2 days and anthesis is shorter as compared to other studied mistletoes species.
P. calyculatus, 5 days (Azpeitia & Lara 2006), P. schiedeanaus, 6 days (Ramírez & Ornelas 2010), P. cordatus (=bicalyculatus) and P. robustus, 3 days (Leal et al. 2006; Guerra et al. 2014), and Ligaria cuneifolia, 4 days (Rivera et al. 1996). Our manual pollinations revealed that P. auriculatus was fully self-compatible, which is the breeding system reported for the New World Ligaria cuneifolia (Rivera et al. 1996), Tristerix corymbosus (Aizen 2005) and three Psittacanthus species (Azpeitia & Lara 2006; Ramírez & Ornelas 2010; Guerra et al. 2014). The observed reproductive system in P. auriculatus flowers allows self- and cross-pollination; however, protandry, male-biased nectar production and variation in nectar offered in P. auriculatus flowers are traits that promote cross-pollination and reduce geitonogamy (see also Fisogni et al. 2011).

The temporal separation of male and female phases (protandry) that takes place in P. auriculatus flowers within 24 h has a slight overlap between both phases (incomplete dichogamy, partial protandry; Lloyd & Webb 1986; Mallick 2001). This incomplete protandry permits self-fertilisation in P. auriculatus flowers if pollen is not removed during the first day of anthesis. In this case, protandry is said to be facultative, as the extent of overlap between sexual forms will depend on the frequency of pollinator visitation and the rate of pollen removal (Mallick 2001). At opening, stigma and anthers of P. auriculatus remain erect and in close proximity, and in subsequent hours, the stigma begins to separate outwards, a movement that might also be triggered by pollen-collecting honeybees (personal observation). The movement could facilitate the contact of stigma with the hummingbird’s head promoting cross-pollen transfer during higher stigma receptivity and avoiding self-interference, but further experimental studies are needed to support this hypothesis.

Fruit set did not differ between hand-, self- and cross-pollinated flowers, but these treatments set significantly fewer fruits than flowers naturally exposed to flower visitors, despite high rates of visitation by pollen and nectar robbers and thieves. This suggests that fruit production was not pollen-limited in the studied population during 2013. In contrast, Guerra et al. (2014) found that flowers of P. robustus naturally exposed to flower visitors set significantly fewer seeds than flowers exposed to hand-, self- and cross-pollination. They suggested that the limitation observed in the reproductive success of P. robustus was not related to pollinator scarcity, but the high frequency of visitation by territorial hummingbirds favoured geitonogamy. During our observations we observed no defence behaviour or hummingbird territoriality to P. auriculatus plants, suggesting that the relatively short longevity of P. auriculatus flowers (as compared with other Psittacanthus species) and the non-territorial foraging behaviour of the hummingbirds observed could play a role in restricting gene flow among neighbouring plants. It was expected that fruit set of flowers after manual cross-pollination and natural pollination should be the same or higher in flowers that were manually cross-pollinated, assuming best pollination conditions (Bierzychudek 1981). Likely, the way the experiment was performed could explain the observed lower fruit production of the manually cross-pollinated flowers (Waser 1978; Stephenson 1979; Bierzychudek 1981; Wyatt 1983), as pollen accumulation by hummingbird flower re-visitation was not incorporated in our experimental design. As shown for New Zealand Peraxilla mistletoes (Robertson et al. 2005), a flower depends on different pollinators (birds and bees) to achieve the pollen load required to achieve successful pollination. Flowers of P. auriculatus exposed to natural pollination could be receiving successive pollen loads from different pollen donors over time, but this was not the case on the manual cross-pollination treatment, in which large pollen loads were applied once 24 h after opening.

The pollination experiment conducted to assess the contribution of autogamy to fruit production showed similar fruit set for both emasculated and intact flowers. This suggests that during the year 2014 for the population under study, natural pollination was effective and autonomous self-pollination did not contribute to female reproductive success. However, the results of the 2013 pollination experiment from the autonomous self-pollination treatment, suggest that in the absence of pollinator visits, autogamy has the potential to contribute to reproduction. Future experimental studies controlling the frequency of pollinator visitation would be helpful to test whether or not autogamy provides reproductive assurance under natural conditions (Eckert & Schaefer 1998).

**Nectar secretion dynamics**

Nectar of P. auriculatus flowers excluded from pollinators is offered at concentrations from 18–32 °Brix, and the volume after 24 h of accumulation (day 2) is ca. 31 μl, with 10 mg
sugar per flower. Both nectar volume and sugar concentration are in the range documented for hummingbird-visited flowers (Ornelas et al. 2007) and Psittacanthus species studied in detail. *P. cordatus* (=bicalyclus) and *P. calycinus* flowers produce ca. 18–22 μl per day (Azpeitia & Lara 2006; Leal et al. 2006), *P. schiedeanus* flowers about 17 μl after 24 h of accumulation (Ramírez & Ornelas 2010), and *P. robustus* produce ca. 90–120 μl per flower at the beginning of anthesis (Guerra et al. 2014). The floral tube (petals partially forming a nectar chamber) of *P. auriculatus* flowers (19.6 mm) is larger than in *P. schiedeanus* (8 mm; Ramírez & Ornelas 2010) but smaller than those of flowers of *P. robustus* (20–30 mm; Guerra et al. 2014), in accordance with the positive correlation between nectar production and corolla length for hummingbird-visited flowers (Ornelas et al. 2007).

Nectar production in *P. auriculatus* seemed to occur in two pulses, at opening and then replenished overnight (between 17:00 h and 08:00 h), after which production ceased. The first pulse of nectar production coincides with the maximum pollen availability, ensuring that most pollen is removed during the first visits on day 1 (all anthers are dehiscent), with almost no overlap between sexual phases (e.g. Mallick 2001) and the highest stigma receptivity on day 2 in which removed nectar is replenished. Flowers of *P. auriculatus* produced additional nectar after repeated removal but were conservative in the secretion of additional sugar (Orndano & Ornelas 2004), meaning that pollinator visitation on day 1 does not stimulate sugar production. Hummingbird-visited plant species respond positively or negatively to repeated nectar removal and the type of response varies even within the same genus (Galleta & Bernardello 1993; Castellanos et al. 2002; Orndano & Ornelas 2004). In Loranthaceae, positive effects of nectar removals were reported in *P. schiedeanus* (Ramírez & Ornelas 2010) and *Ligaria cuneifolia* (Rivera et al. 1996), and the effects of nectar removal on nectar production were not significant in *P. robustus* flowers (Guerra et al. 2014). Also, the sum of sugar secreted by flowers after repeatedly removing the nectar during the first 2 days was 22% lower than sugar production in flowers excluded the same amount of time without repeated removals. From these results we infer active nectar re-absorption, that nectar solutes decreased in undisturbed flowers (Búrquez & Corbet 1991), minimising costs of nectar production for the female phase when pollinators do not remove nectar during the male phase (Orndano & Ornelas 2004). As nectar production is costly in terms of growth and seed production (Pyke 1991; Ordano & Ornelas 2004), 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 into other functions (Nicolson 1995; Nepi et al. 2001; Pacini et al. 2003; Willmer 2011) and/or to the development of seeds and fruits (Búrquez & Corbet 1991; Orndano & Ornelas 2005). Nevertheless, nectar standing crop data suggest that almost no nectar of the total secreted by flowers of *P. auriculatus* was available for re-absorption due to the high rate of hummingbird visits and nectar robbing by bumblebees, Bombus, butterflies, wasps and ants.

**Pollination syndromes within functional groups**

The role of hummingbirds acting as main pollinators of *P. auriculatus* flowers was supported by our data. However, an experimental approach is needed to determine the abundance and effectiveness of the various floral visitors as pollinators of *P. auriculatus*, and the effectiveness of the various hummingbird species in cross-pollinating *P. auriculatus* flowers throughout the flowering period. The ornithophilous syndrome, as originally conceived (Faegri & Van der Pijl 1979; Fenster et al. 2004), included various functional groups of bird pollinators that hover (hummingbirds and honeyeaters) or perch (sunbirds or orioles) while accessing nectar. The conceptual framework of the hummingbird pollination syndrome, often associated with diurnal tubular red or orange flowers, typically ignores other floral traits, and morphological and behavioural traits of pollinators seemly linked to a more specialised hummingbird pollination syndrome. The study of traits associated with nectar production patterns (e.g. Ornelas et al. 2007), prostandry (Bertin & Newman 1993), flower longevity and orientation (e.g. Tadey & Aizen 2001) and variation in foraging behaviour among hummingbird species might help better characterise plant species exclusively pollinated by hummingbirds. The floral characteristics of *P. auriculatus* correspond to the typical suite of characteristics associated with ornithophilous pollination within Loranthaceae, including odourless, diurnal, tubular, red or orange flowers with robust corollas and sexual organs that must resist the effects of repeated contact with the heads of birds accessing nectar (Calder & Bernhardt 1984). As compared with other *Psittacanthus* species, a case of a more specialised pollination in *P. auriculatus* would involve several floral traits, especially those related to the shape (curled versus non-curled petals), colour and longevity of the flowers, partial prostandry and a diurnal nectar production pattern. This suite of traits suggests at least that the pollination syndrome can be extended within the same functional group of pollinators, and that a transition from a more generalised pollination syndrome that includes hummingbirds and passerines acting as potential pollinators, as observed in *P. schiedeanus* (Ramírez & Ornelas 2010) and *P. robustus* (Guerra et al. 2014), to a more specialised hummingbird pollination syndrome has occurred within this clade in *P. auriculatus*.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:
REFERENCES


Benton T.M., Cracraft J., Ornelas J.F., Ordano M., de-Nova A.J., Quintero M.E., Perez-Crespo, Ornelas, Martín-Rodríguez, González-Rodríguez & Lara (2016) Figure S1. Amount of nectar of 3-day-old Psittacanthus auriculatus flowers removed at 3-h intervals. (A) Nectar volume. (B) Amount of sugar. N = 10 plants, n = 51 flowers. Letters indicate mean pair differences after Games-Howell’s multiple comparisons.

Benton T.M., Cracraft J., Ornelas J.F., Ordano M., de-Nova A.J., Quintero M.E., Perez-Crespo, Ornelas, Martín-Rodríguez, González-Rodríguez & Lara (2016) Figure S2. Comparison between the amounts of nectar produced by Psittacanthus auriculatus flowers after 24 h of accumulation and repeated removal (five removals every 3 h) over 24 h since flower opening. Mean ± SE.