

The cost of nectar replenishment in two epiphytic bromeliads

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(Accepted 17 February 2005)

Abstract: Animal-pollinated angiosperm plants that replenish removed nectar invest energy that can entail a reproductive cost. Here we investigated whether or not seed production is affected by replenishing nectar in hummingbird-pollinated *Tillandsia multicaulis* and *T. depeana* (Bromeliaceae) in a montane cloud forest in eastern Mexico. These epiphytic plants respond strongly and positively to repeated nectar removal. The female reproductive cost was assessed in manually, cross-pollinated flowers with or without repeated nectar removal. Seed production from experimental flowers was then contrasted with those naturally exposed to pollination and nectar removal. *Tillandsia depeana* set the same number of seeds of the same size regardless of whether or not it had to replenish nectar. Seeds were slightly smaller if the result of natural open pollination than from copious hand crossing. In contrast, *T. multicaulis* set about half as many seeds when it had to replenish than when it did not, indicating a substantial cost to replenishment. There was no difference in seed number of *T. multicaulis* between open and hand-augmented pollination. The contrasting results for plants that respond strongly and positively to repeated nectar removal suggest that the female reproductive costs of nectar replenishment can range from costly to beneficial, depending on the conditions of the plants and on the species.

Key Words: Bromeliaceae, cloud forest, epiphytes, hummingbirds, Mexico, nectar cost, nectar removal, seed production, *Tillandsia*

INTRODUCTION

It is broadly assumed that nectar production has a female reproductive cost. Investment in nectar production can represent up to 30% of the energy devoted by a flower in *Asclepias quadrifolia* (Pleasants & Chaplin 1983) and 33% of the photosynthates assimilated by *A. syriaca* plants (Southwick 1984), and caused a significant seed number reduction in plants of *Blandfordia nobilis* subjected to repeated nectar removal (Pyke 1991). However, nectar investment estimated in *Pontederia cordata* represented only 3% of the floral tissue and it was 45% higher than expected to assure bee pollination (Harder & Barrett 1992). Nectar production was not correlated with vegetative or reproductive traits in *Echium vulgare*, and hence no costs of nectar production were detected (Leiss *et al.* 2004). In *Prosopis glandulosa* var. *torreyana* the nectarless morph does not differ from the nectar

morph in terms of vegetative growth (Golubov *et al.* 2004), suggesting that the cost of nectar production is negligible or that resources allocated to growth are different from those allocated for reproduction. Nectar replenishment can then range from costly to beneficial depending mainly on the identity of the pollinators and changes in their abundance, and the habitat and breeding system of the plant (McDade & Weeks 2004, Ordano & Ornelas 2004). Nonetheless, the effect of nectar replenishment on female reproduction depends on the level at which reproduction is analysed because resource allocation pathways are different at the branch, individual plant, fruit and seed levels (Obeso 2004). Consequently, there is conflicting evidence in terms of reproductive fitness for the broadly assumed expenses in nectar production. There is also some convincing evidence that nectar reabsorption and plasticity for the scheduling of nectar production represent nectar-saving mechanisms in response to stigmatic pollen deposition or pollen removal (Búrquez & Corbet 1991, Luyt & Johnson 2002, Nepi *et al.* 2001), which may result from selective

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pressures imposed by nectar production costs, mating systems and/or flower visitors (Ordano & Ornelas 2004 and references therein).

In the cloud forests in Veracruz, Mexico, *Tillandsia deppeana* and *T. multicaulis* (Bromeliaceae) have diurnal anthesis and are pollinated mainly by hummingbirds. These systems are particularly well suited to investigate the effects of replenishing removed nectar on seed production for several reasons. (1) These bromeliads are monocarpic and produce many seeds per fruit (García-Franco & Rico-Gray 1991), so pollen receipt and pollen delivery by pollinators is important for their reproductive success. (2) The plants are non-autogamous self-compatible but benefit from producing few flowers at day and thus reducing chances for geitonogamous crosses. (3) Both *Tillandsia* species produce copious nectar and had the highest rate of nectar replenishment amongst species studied to date (Ordano & Ornelas 2004). (4) Neither *Tillandsia* species reabsorbs nectar or possesses an obvious mechanism to save energy from nectar secretion after stigmatic pollen deposition (Ordano & Ornelas 2004). (5) Although tank bromeliad species are portrayed as environmentally stressed due to their epiphytic habit (Benzing 1998, Nadkarni & Solano 2002), they can accumulate water and nutrients in their tanks (Benzing 1980). An exploratory meta-analysis revealed more replenishment of nectar (highest effect sizes) for species inhabiting wet tropical habitats (Ordano & Ornelas 2004). Thus, these hummingbird-pollinated species are a good model for examining reproductive costs and relationships among energy allocation to nectar production.

To determine the effects of replenishing removed nectar on seed production, we addressed two questions: (1) Is replenishing nectar costly or beneficial in terms of seed production? (2) Does experimentally increased nectar replenishment affect biomass allocation to seeds?

METHODS

Study area

The study area was the fragmented landscape of tropical montane cloud forests near Xalapa City, Veracruz, Mexico. A full description of the area and vegetation is given by Castillo-Campos (1991). Fieldwork was carried out from July 2002 to June 2004 around the Jardín Botánico ‘Francisco X. Clavijero’ (19°30'N, 96°56'W; at 1350 m altitude).

Study species

Tillandsia multicaulis Steudel (Bromeliaceae) is an abundant epiphytic tank bromeliad one fifth to one

third the size of *T. deppeana* inhabiting montane forests (1200–1900 m altitude) from Mexico to Panama (Smith & Downs 1977). In the study area, flowering ranges from July to October. Each plant produces up to seven inflorescences and 6–38 tubular hermaphroditic flowers that last nearly 2 d (Ordano & Ornelas 2004). The bracts are red and flower colour is uniformly violet. It is visited by hummingbirds and by at least two pollen-collecting bee species.

Tillandsia deppeana Steudel is a non-abundant endemic tank bromeliad found in pine and tropical deciduous forests (1080–1800 m altitude) from Tamaulipas to Oaxaca, Mexico (Smith & Downs 1977). This monocarpic, rosette-forming bromeliad (0.8 m high) is characterized by reddish, pinnate inflorescences. Hermaphroditic flowers with blue or blue-white tubular corollas are open for 1 d. This species is self-compatible (but not autogamous), and flowering occurs from January to June in the study area. Each plant produces 70–230 flowers and opens *c.* 3 flowers d⁻¹ (Ordano & Ornelas 2004). It is visited by hummingbirds, bees and butterflies. Both *Tillandsia* species host flower mites. *Tillandsia* species typically sprout ramets during reproduction, after which the reproductive genet dies. The cost of nectar may differ between species due to differences in the mode and frequency of sprouting ramets. Although sprouting was observed for *T. deppeana*, most flowering plants were solitary (95%) or have lost sprouts by the time of flowering, whereas for *T. multicaulis* *c.* 35% of reproductive individuals present at least a non-reproductive ramet (M. Ordano & J. F. Ornelas, pers. obs.). Assuming that ramets will have future reproductive chances (J. G. García-Franco, pers. comm.), the costs of replenishing removed nectar in *T. multicaulis* should be relaxed among individuals with ramets.

Field procedures

The nectar-removal experiment with *T. multicaulis* was carried out by carefully removing plants before flowering from the surroundings of Xalapa, and then transporting and transplanting them to new host trees in a montane cloud forest remnant near our laboratory. We frequently watered them until the beginning of the experiment. The experiment with *T. deppeana* was carried out with plants naturally growing on various host tree species located at the Jardín Botánico and Parque Ecológico ‘Francisco X. Clavijero’. Using transplanted versus naturally growing plants resulted from our logistic capacity to work with epiphytic bromeliads in the field and species differences in plant abundance in the field. We bagged the whole inflorescence and fully developed buds with tulle to exclude floral visitors the evening before nectar measurements. Flower mites were excluded from

manipulated flowers by applying tanglefoot (sticky resin; Tanglefoot Co., Grand Rapids, Michigan, USA) as they may consume up to half the nectar otherwise available to pollinators and induce additional nectar secretion (Lara & Ornelas 2001). Nectar was extracted carefully the day after the exclusion with capillary tubes without removing the flowers from the plants. Nectar volume was measured using graduated micropipettes (5–10 μ l) and a ruler; sugar concentration (percentage sucrose) with a pocket refractometer (American Optical 10431, Buffalo, New York, USA; range concentration 0–50°, BRIX units), and the amount of sugar produced was expressed as milligrams according to Bolten *et al.* (1979). Effects of nectar removal on total nectar production had been tested before and both species replenish removed nectar (Ordano & Ornelas 2004). Flowers with the highest removal intensity (4–5 removals) produced more than three times the amount of nectar over time than those with the lowest removal intensity (one removal). The amount of sugar also increased over threefold with increased removal intensity in both *Tillandsia* species (Ordano & Ornelas 2004). Here we used the same manipulation intensity to stimulate nectar replenishment in both species.

Effects of replenishing nectar on seed production

Plants of both species were subjected to one of three treatments: (1) some were repeatedly emptied of nectar and hand cross-pollinated; (2) others were probed with a plugged micropipette in a similar way and similarly cross-pollinated by hand; and (3) yet others were left to be naturally pollinated. The first two groups were excluded from floral visitors as described above. The treatment in which plugged micropipettes are inserted into the flower but no nectar is removed acts as a control for the effect of pipette insertion into the flower (Castellanos *et al.* 2002), and how such insertion might affect nectar and seed production. The flowers of the third group were unbagged and exposed to natural levels of pollination and nectar removal by hummingbirds and flower mites potentially brought by hummingbird bills. The natural, unbagged treatment acts as a 'pseudo-control' for seed production, although the mechanisms affecting seed production in this treatment are unclear and may be due either to resource allocation or pollen load, or both. In *T. multicaulis* (July–October 2002), nectar was extracted twice a day from individual flowers (N = 15 plants, 58 flowers), with two removals the first day (at 09h00 and 15h00) and two removals during its second day (09h00 and 13h00). In *T. deppeana* (February–May 2003), nectar was extracted five times from individual flowers (N = 11 plants, 102 flowers), at 3-h intervals during the day (07h00 to 19h00). Flowers remained

bagged between removals or insertions with plugged micropipettes. In a previous report we found that the magnitude of the response (nectar replenishment) was not influenced by the magnitude of perturbation (number of removals) or flower longevity as a measure of duration of experimental manipulation (Ordano & Ornelas 2004).

The same number of insertions with plugged micropipettes was applied to control flowers on each species (N = 16 plants, 60 flowers for *T. multicaulis*; N = 10 plants, 80 flowers for *T. deppeana*). We assumed that micropipette insertion has a negligible effect on nectar replenishment and properly simulates the probing behaviour of pollinators. In total, 158 flowers were not manipulated and exposed to natural levels of pollination and nectar removal (N = 22 plants, 85 flowers for *T. multicaulis*; N = 14 plants, 73 flowers for *T. deppeana*). Each plant received a single treatment.

Flowers subjected to repeated micropipette insertions (with or without removal of nectar) were manually cross-pollinated after the first micropipette insertion by smearing the anther from the donor onto the receptive virgin stigma with pollen grains directly from anthers from randomly selected plant donors. All pollen deposition occurred during periods of stigma receptivity. Pollen grains deposited on individual stigmas by hand-pollination were not counted, but pollen loads were enough to fertilize most ovules; a stigma almost covered with easily visible yellow pollen could have over 3000 pollen grains (M. Ordano & J. F. Ornelas, pers. obs.). Both *Tillandsia* species have flowers with many ovules (mean \pm SE, *T. multicaulis* = 429 \pm 8.7 ovules per flower; *T. deppeana* = 268 \pm 2.6 ovules per flower), so multiple visits may be necessary to saturate stigmas and allow selective fertilization by high-quality pollen. Pollinator visitation rates are not available for these species. However, current work on pollen receipt and nectar standing crop, as indirect measures of pollinator visitation rates, indicate that stigmas are heavily loaded with conspecific pollen (mean \pm SE, *T. multicaulis* = 807 \pm 88 pollen grains per stigma, N = 10; *T. deppeana* = 3040 \pm 129 pollen grains per stigma, N = 10), and more than 96% of pollen grains have germinated in both species by late afternoon. Preliminary data on nectar standing crops from the same flowers suggest low visitation rates and/or high pollinator efficiency in *T. multicaulis* when nectar standing crops (mean \pm SE, *T. multicaulis* = 34.2 \pm 1.6 μ l per flower, N = 10; *T. deppeana* = 11.9 \pm 1.2 μ l per flower, N = 10) are compared with nectar volumes from bagged flowers (mean \pm SE, *T. multicaulis* = 3.3 \pm 0.1 μ l per flower, N = 31; *T. deppeana* = 4.1 \pm 0.1 μ l per flower, N = 37; Ordano & Ornelas 2004). Altogether these preliminary data suggest that pollinator visitation rates are lower for *T. multicaulis*, and that flowers of both species are experiencing pollen saturation on their stigmas.

Individual plants received a unique treatment or control, and randomly selected flowers from each individual plant represent the sampling unit. Fruits from experimental flowers were collected *c.* 6 mo later). Seeds of each fruit were counted by eye and weighed (seed mass) to the nearest 0.01 mg.

Statistical analyses

Total seed mass was highly dependent on seed number (*T. multicaulis*, adjusted $r_s = 0.81$, $F_{1,27} = 121$, $P < 0.0001$; *T. deppeana*, adjusted $r_s = 0.87$, $F_{1,73} = 487$, $P < 0.0001$). The residuals of this relationship were calculated for both species as an estimation of biomass allocation per seed (EBS), independently of seed number variation. To evaluate the effects of nectar replenishment after repeated removal on seed production, we used univariate nested ANOVAs with Type III sum of squares on seed number and EBS. In the model, treatment (insertions with nectar removal and insertions with plugged micropipettes) was a fixed factor and plant was nested within treatment. Seed production of flowers exposed to natural pollination was then contrasted with that from experimental flowers using post hoc mean comparisons (Games–Howell procedure, $\alpha = 0.05$). Our data met assumptions of normality (Kolmogorov–Smirnov test, $P > 0.2$) and homogeneity of variance (Levene’s test, $P > 0.05$). Raw data (mean \pm SE) are reported. Flowers of a given plant were all bagged but not all treated. It is likely that plants re-allocated a proportion of available resources from the non-treated flowers to the treated flowers. Bagging all non-treated flowers should have minimized this possible confounding factor because these flowers are non-autogamous and were not pollinated at all. Nonetheless, we consider that our nested experimental design accounted for variation due to plant traits. Analyses were run on Statview and SuperANOVA (Abacus Concepts, Inc., Berkeley), and Statistica 5.5 (StatSoft, Inc., Tulsa).

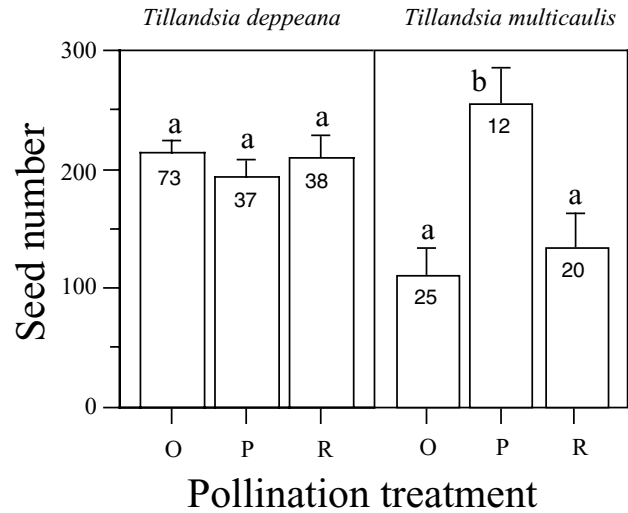


Figure 1. Effect of replenishing removed nectar on seed number (mean \pm SE) in *Tillandsia deppeana* and *T. multicaulis* (Bromeliaceae). Flowers with repeated nectar removal (R) and repeated insertions with plugged micropipettes (P) were manually cross-pollinated and excluded from flower visitors. O = flowers exposed to natural levels of pollination and nectar removal. Numbers within bars indicate sample size (number of fruits per treatment) and sample size differences were due to missing values. Letters indicate differences between groups after post hoc mean contrasts ($P < 0.01$).

RESULTS

Effects of replenishing nectar on seed production

Repeated nectar removal affected subsequent seed production differently in both *Tillandsia* species (Figure 1). In *T. multicaulis*, seed number varied significantly with treatment, decreasing with treatment of micropipette insertions and nectar removal, and EBS did not differ between treatments (Table 1). Fruits from manually cross-pollinated flowers with plugged micropipette insertions produced almost twice as many seeds (mean \pm SE: 256 ± 26.3) than those subjected to repeated

Table 1. Summary of univariate nested ANOVAs on the effects of replenishing removed nectar on seed production in *Tillandsia multicaulis* and *T. deppeana*. Two pollination treatments (with or without repeated nectar removal) applied to plants manually cross-pollinated. EBS, estimated biomass per seed. Individual plants were nested within treatment.

	Pollination treatment			Plant (pollination treatment)			Error df
	df	F	P	df	F	P	
<i>Tillandsia multicaulis</i>							
Seed number	1	5.56	0.032	12	1.23	0.350	15
EBS	1	0.085	0.775	12	1.32	0.302	15
<i>Tillandsia deppeana</i>							
Seed number	1	0.948	0.334	15	2.40	0.009	58
EBS	1	0.424	0.518	15	3.36	0.0005	58

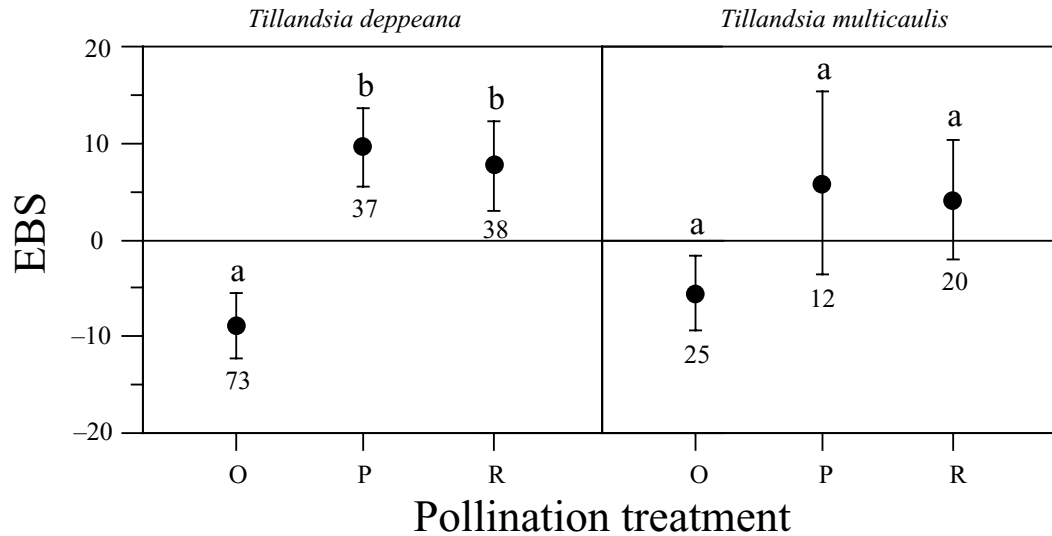


Figure 2. Effect of replenishing removed nectar on estimated biomass per seed (EBS) in *Tillandsia deppeana* and *T. multicaulis* (Bromeliaceae). EBS values (mean \pm 1 SE) are the residuals after regressing seed number against total seed mass. Flowers with repeated nectar removal (R) and repeated insertions with plugged micropipettes (P) were manually cross-pollinated and excluded from flower visitors. O = flowers exposed to natural levels of pollination and nectar removal. Numbers indicate sample size (number of fruits per treatment) and sample size differences were due to missing values. Letters indicate differences between groups after post hoc mean contrasts ($P < 0.01$).

nectar removal (135 ± 26.2 ; Table 1). Flowers exposed to natural visitation produced 113 ± 20.4 seeds (Figure 1). EBS was not significantly different among flowers subjected to insertions with plugged micropipettes, flowers subjected to repeated nectar removal, and those exposed to natural levels of pollination and nectar removal (Table 1, Figure 2).

In contrast, nectar removal had no significant effect on *T. deppeana* seed production (Table 1, Figure 1). Individual plants (nested within treatment) were significantly heterogeneous for each dependent variable (Table 1). On average, flowers subjected to repeated nectar removal produced more seeds (mean \pm SE: 210 ± 16.3) than those subjected to insertions with plugged micropipettes (194 ± 12.6), but these differences were not statistically significant (Table 1). Flowers exposed to natural levels of pollination and nectar removal produced 214 ± 10.4 seeds per fruit (Figure 1). The EBS was not significantly different between groups of treated flowers (Figure 2). However, significant differences were observed between treated flowers and those exposed to natural levels of pollination and nectar removal. EBS values from treated flowers were significantly higher when contrasted with those from untreated flowers (Figure 2).

DISCUSSION

Nectar removal effects variation

This study assessed how nectar replenishment affects seed production by experimentally removing nectar from

manually cross-pollinated flowers. On average, removing nectar reduced seed production in *Tillandsia multicaulis* by nearly 50%. Additional nectar secretion in flowers subjected to repeated nectar removal (2.4 ± 0.1 mg of sugar per flower) is maternally expensive considering that flowers without subsequent nectar removal produce on average 1.28 ± 0.04 (SE) mg of sugar following anthesis (Ordano & Ornelas 2004). This indicates that the production of an additional 1 mg of sugar after repeated removal might have halved female reproductive fitness. In contrast, additional nectar production in *T. deppeana* flowers subjected to the same removal treatment had no effect in terms of seed production.

We investigated whether nectar replenishment is costly for flowers in terms of seed production. There are few previous studies with this approach and the evidence up to now is inconclusive. Previous studies have shown that the expenses of nectar production are negligible in terms of investment in floral tissue or vegetative growth (Golubov *et al.* 2004, Harder & Barrett 1992, Leiss *et al.* 2004), or reasonably high in terms of energy investment or photosynthate assimilation (Pleasants & Chaplin 1983, Southwick 1984) or seed production (Pyke 1991). Here, we have shown that the cost of replenishing removed nectar in terms of seed production, varied from high to negligible between *Tillandsia* species.

Flowers of *T. multicaulis* exposed to natural levels of pollinators and nectar removal produced the same number of seeds as those bagged with repeated nectar removal, but fewer seeds than flowers that were manually cross-pollinated and received micropipette insertions but were not emptied of nectar. This result suggests that plants

might be resource limited and flower visitors (pollinators, flower mites) would be extracting similar amounts of nectar as the amount removed experimentally. Without an examination of the number of pollen grains deposited on the stigmas of these naturally-pollinated flowers, the lower seed set for this treatment being a consequence of less pollen being deposited on stigmas by natural pollinators, in contrast to the hand pollinations, is not guaranteed. Likewise, pollen receipt from the same plant and deleterious consequences of inbreeding might in part explain the lower seed set in this treatment. This was not the case for *T. deppeana*; seed production in manual crosses equaled that of flowers exposed to natural conditions, suggesting that this species was not resource limited. By transplanting *T. multicaulis* plants on to new hosts, one could argue that they were subjected to increased environmental stress. Therefore, costs of nectar production would be evident under a scenario of resource limitation (Obeso 2002), in which transplanted plants had fewer resources on their new hosts. Lastly, decreased seed production in *T. multicaulis* could be associated with the lower physiological efficiency of smaller-sized epiphytic bromeliads (Laube & Zotz 2003, Schmidt & Zotz 2001). *Tillandsia deppeana* plants are nearly five times larger than those of *T. multicaulis*, and larger bromeliad plants could accumulate more water and nutrients in their tanks. Nevertheless, due to confounding factors such as plant transplantation, alternative hypotheses for comparisons between species require further testing.

Conclusions and implications

Nectar production costs have been investigated for only a few species (Golubov *et al.* 2004, Harder & Barrett 1992, Leiss *et al.* 2004, Pleasants & Chaplin 1983, Pyke 1991, Southwick 1984, this study). These studies have shown that the female reproductive costs of nectar replenishment are not universal. Although flowers of *Tillandsia* plants replenish both volume and sugar after repeated removal (Ordano & Ornelas 2004), the adaptive function of replenishment, if any, needs further research. Here we only examined the effect of nectar replenishment on female fitness, and the effect of replenishment on male fitness awaits investigation. High replenishment rates would be potentially advantageous in these monocarpic plants, maximizing pollen movement and consequently male and female reproductive success. It is possible that nectar secretion might enhance pollen export (male function) even after stigmatic pollen deposition. Male reproductive success may benefit in two ways. First, pollinators leaving spatially isolated plants with few available open flowers from flowers with copious nectar could fly longer distances to subsequent isolated epiphytic plants, which could result in pollen moving farther and

reduce geitonogamy. Second, male reproductive success may increase if pollinators revisit the same flower after encountering conspecific flowers and depositing pollen grains removed in their first visit to that flower promoting pollen competition and quality-based female choice of donors. Even if nectar is costly, if plant reproductive success increases with increasing pollen deposition, for example if maternal plants can choose high-quality pollen donors from the pollen pool available, plants would be selected to replenish nectar after removal by pollinators. Our data clearly revealed a need for more studies on the effects of nectar production on fruit- and/or seed-set.

ACKNOWLEDGEMENTS

We thank C. González, J. C. López, J. Tolome, A. Martínez Virués, A. Cruz, L. Jiménez, M. Carrillo, A. Elrick, and M. Lisman for help in fieldwork and logistics; A. Flores, A. Cruz, J. González Astorga, and A. Hernández for stimulating discussion; Renate A. Wasselingh, Paul Wilson, and an anonymous reviewer for constructive suggestions on previous versions. Research support was provided by the Departamento de Biología Evolutiva (Ref. 902-11-563), Instituto de Ecología, A.C., Mexican Government through the Instituto Mexicano de Cooperación Internacional (IMEXCI) of the Secretaría de Relaciones Exteriores (SRE), and Organization of American States Fellowship Program (LASPAU) of the Academic and Professional Programs for the Americas. This work constitutes partial fulfillment of M. Ordano's doctorate in Ecología y Manejo de Recursos Naturales at the Instituto de Ecología, A.C.

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