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Nectar removal effects on seed production in *Moussonia deppeana* (Gesneriaceae), a hummingbird-pollinated shrub[1]

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Abstract: Animal-pollinated angiosperm plants that respond positively to nectar removal by replenishment invest energy that can entail a reproductive cost. Here we use nectar removal manipulation to investigate whether nectar removal increases total nectar production in *Moussonia deppeana* (Gesneriaceae) and to see if this leads to lower seed production. We found that flowers strongly respond to increased nectar removal by producing 2–4 times as much additional nectar. Other nectar removal treatments had little effect on nectar production and rule out alternative explanations at the flower level. Compared with open-pollinated flowers, hand cross-pollination yielded similar seed production in flowers with experimentally increased nectar removal. Although flower-level effects of nectar replenishment on seed production were not detected in *M. deppeana*, costs of nectar replenishment need further study as tradeoffs between nectar production and seed production could be expressed at the plant level and/or as long-term effects.
Keywords: Gesneriaceae, nectar production, nectar removal, nectar replenishment, protandry.

Résumé : Les plantes angiospermes à pollinisation par les animaux qui répondent positivement au retrait de nectar par le réapprovisionnement investissent de l'énergie ce qui peut entraîner un coût reproductif. Dans une expérience de manipulation, nous avons utilisé le retrait de nectar afin d'étudier si le retrait cause une augmentation de la production total de nectar chez *Moussonia deppeana* (Gesneriaceae) et pour vérifier si cela conduit à une diminution de la production de graines. Nous avons observé que les fleurs répondent fortement à l'augmentation du retrait de nectar en produisant de 2 à 4 fois plus de nectar. Les autres traitements de retrait de nectar avaient peu d'effets sur la production de nectar ce qui exclut les explications alternatives au niveau de la fleur. Pour les fleurs ayant subit une augmentation expérimentale du retrait de nectar, la pollinisation croisée manuelle résultait en une production de graines similaire à celle des fleurs à pollinisation libre. Même si aucun effet du réapprovisionnement en nectar sur la production de graines n'a été détecté au niveau de la fleur chez *M. deppeana*, il est nécessaire de poursuivre l'étude des coûts du réapprovisionnement en nectar puisque des compromis entre la production de nectar et de graines peuvent exister au niveau de la plante ou à plus long terme.
Mots-clés : Gesneriaceae, production de nectar, protandrie, réapprovisionnement en nectar, retrait de nectar.

Nomenclature: Wiehler, 1975; 1982.

Introduction

Documentation of the effects of nectar removal on subsequent nectar production is interesting from a plant–pollinator interaction perspective. Flowers of several plant species replenish both volume and sugar after repeated removal. Previous studies have shown that experimental nectar removal typically increases total nectar production (reviewed in Castellanos, Wilson & Thomson, 2002; Ordano & Ornelas, 2004), but the adaptive function, if any, needs further research. Flowers pollinated over a long period of time would benefit by replenishing removed nectar (at least water in nectar) when plants are subject to low pollinator visitation rates, particularly in environments where nectar tends to evaporate (Castellanos, Wilson & Thomson, 2002). Replenishment of sugar and water may be favoured if male or female reproductive success increases with an increased number of pollinator visits or number of probes by the pollinator (Mitchell, 1993). Under those circumstances, high replenishment rates would be potentially advantageous, maximizing pollen movement and consequently male and female reproductive success (see also Ordano & Ornelas, 2005). Lastly, low replenishment rates may encourage pollinators to revisit plants while keeping the rate of geitonogamy low (Harder & Barrett, 1996). Animal-pollinated angiosperm plants that respond positively to nectar removal by replenishment invest energy that can entail a reproductive cost (reviewed in Castellanos, Wilson & Thomson, 2002; Ordano & Ornelas, 2004). There are few studies investigating nectar production costs. Previous studies have shown that the expenses of nectar produc-

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tion are negligible in terms of investment in floral tissue or vegetative growth (Harder & Barrett, 1992; Golubov *et al.,* 2004; Leiss, Vrieling & Klinkhamer, 2004), but reasonably high in terms of energy investment, photosynthate assimilation (Pleasants & Chaplin, 1983; Southwick, 1984), or seed production (Pyke, 1991; Ordano & Ornelas, 2005). Yet we know little about the reproductive costs of nectar replenishment. Ordano and Ornelas (2005) investigated whether or not seed production was affected by replenishing nectar in two bromeliad hummingbird-pollinated *Tillandsia* species that respond strongly and positively to repeated nectar removal (Ordano & Ornelas, 2004). *Tillandsia deppeana* set the same number of seeds of the same size regardless of whether or not it had to replenish nectar. In contrast, *T. multicaulis* set about half as many seeds when it had to replenish as when it did not. These contrasting results suggest that the female reproductive costs of nectar replenishment can range from costly to beneficial depending on the identity of the pollinators and changes in their abundance, the habitat and breeding system of the plant, and the level at which reproduction is analyzed (McDade & Weeks, 2004; Ordano & Ornelas, 2004; 2005). Consequently, there is conflicting evidence in terms of reproductive fitness for the broadly assumed expenses in nectar production.

Here, we used the same approach to increase nectar production in *Moussonia deppeana* (Schlecht. & Cham.) Hanst. (Gesneriaceae), a hummingbird-pollinated perennial shrub (Lara & Ornelas, 2003). This system is particularly well suited for an investigation of the effects of replenishing removed nectar on seed production for several reasons. (1) This gesneriad is protandrous and produces many seeds per fruit (Lara & Ornelas, 2003), so effective transfer of pollen is important for its reproductive success. If plant reproductive success increases with increasing pollen deposition, plants would be selected to replenish nectar after its removal by pollinators. (2) The plants are non-autogamous and self-compatible but benefit from producing few flowers at day and thus reducing chances for geitonogamous crosses. (3) Flowers produce copious amounts of nectar compared to bee-pollinated gesneriads (Stiles & Freeman, 1993; Buzato, Sazima & Sazima, 2000). (4) An anther smut fungus infects *Moussonia* plants, modifying floral and reproductive characteristics of the host and the behaviour of its pollinator (Lara & Ornelas, 2003). Diseased plants produce fewer seeds compared to healthy individuals, and infected flowers are retained longer than uninfected ones, producing additional nectar sugar (Lara & Ornelas, 2003), *i.e.,* lower seed production might be due to the energy cost imposed by high nectar production. If increased nectar production reduces seed production in manipulated flowers, increased nectar production by infected flowers may also cause a similar reduction in seed set. Although there is no difference in nectar production rates between infected and uninfected flowers of *Moussonia* during the first 4 d of their lifetimes, infected flowers produced nectar over a longer period of time (Lara & Ornelas, 2003). Other plant species infected with anther smut fungi show reduced nectar production by diseased individuals, *e.g., Viscaria vulgaris* (Jennersten, 1988) and *Silene latifolia* (Shykoff & Bucheli, 1995), and this reduction in attractiveness may lower insect visitation (Alexander, 1990; Jennersten & Kwak, 1991; Shykoff & Bucheli, 1995). (5) Responses to nectar removal manipulation seem to be stronger for species pollinated by hummingbirds inhabiting wet tropical habitats (Ordano & Ornelas, 2004). Thus, this hummingbird-pollinated species is a good model for examining reproductive costs and relationships among energy allocation to nectar reproduction that might contribute to a broader understanding of how fungi exploit pollination systems.

To determine the effects of repeated removal on total nectar production and the effects of replenishing removed nectar on seed production in *Moussonia deppeana*, we addressed the following questions: (1) Do flowers respond positively to repeated nectar removal by increasing total nectar production? And (2) does experimentally increased nectar replenishment affect seed production?

Methods

STUDY AREA AND SPECIES

The study area was the fragmented landscape of tropical montane cloud forests near Xalapa City, Veracruz, Mexico (19° 30' N, 96° 57' W). We worked with a small *Moussonia* population (~ 300 plants) growing in shaded areas of a remnant of cloud forest (29 ha) in the Parque Ecológico Francisco Xavier Clavijero (at 1 225 m asl). Mean annual precipitation is 1 500 mm, and mean annual temperature is 18 °C. The climate is mild and humid throughout the year, with a dry, cold season from November to March. A full description of the area is given by Castillo-Campos (1991). Fieldwork was conducted from January to April in 2002 and 2003.

Moussonia deppeana (Gesneriaceae) (hereafter *Moussonia*) is an abundant, 1- to 3-m-tall perennial subshrub distributed in forests from southern Mexico to Honduras (Wiehler, 1982). Plants grow solitarily and flower from November to April. The axillary inflorescences have pronounced peduncles with compound cymes of 4 flowers each (Wiehler, 1975). On average, plants open 8 flowers daily at mid-flowering season (mean ± SD, 8.01 ± 3.5, $n = 20$; Lara & Ornelas, 2001). The orange-red, tubular flowers (~ 32 mm) are pollinated by the amethyst-throated hummingbird *(Lampornis amethystinus)* (Lara & Ornelas, 2001; 2002). *Moussonia* flowers are protandrous, opening in the morning and staying open 4 d. Each flower passes through a 2-d male period (staminate phase), followed by a 2-d female phase (pistillate phase) (Lara & Ornelas, 2001). More nectar is secreted on average during the staminate phase (mean ± SD, 1.98 ± 1.6 µL·flower^{-1}·d^{-1}, $n = 20$) than in the pistillate phase (1.12 ± 0.5 µL·flower^{-1}·d^{-1}, $n = 20$), and total nectar volume produced by flowers over their lifetimes is on average 27 µL. Sugar concentration (Brix scale) is the same, on average, between flower phases (16% sucrose; Lara & Ornelas, 2003). Fruits are dry, bivalved capsules with loculicidal dehiscence (Wiehler, 1975). Seeds are small (~ 0.5 mm) and numerous (~ 450 seeds per capsule) (Lara & Ornelas, 2002). *Moussonia* plants host the fungus *Fusarium moniliforme* (Deuteromycota: Section *Liseola*) (Lara & Ornelas, 2003). The fungal exploitation of this plant-hummingbird mutualism offers interesting paral-

lels and contrasts with other pollinator-disease transmission systems because diseased plants are not completely sterilized; plants infected by the fungus produce both healthy and diseased flowers. In infected flowers fungal spores replace all pollen in the anthers, and female function (pistillate phase) is aborted. Infected flowers are retained 2 d longer (6 d total) than uninfected ones (4 d), producing an additional 2 $mg \cdot \mu L^{-1} \cdot flower^{-1}$ of nectar sugar (Lara & Ornelas, 2003). Diseased plants produce about twice as many flowers, infected and uninfected, compared to healthy plants over the course of the flowering season, and *ca* 24% (range 1–70%) of the flowers on diseased plants (infection intensity) are smutted. Fungal spores are disseminated among plants by hummingbirds, and hummingbird visitation is about 3 times higher to diseased plants, regardless of flower number and sexual phase (Lara & Ornelas, 2003). It is not known whether the fungus directly affects fruit production. Diseased plants produce the same number of fruits but fewer seeds per capsule than healthy individuals (Lara & Ornelas, 2003). We have not observed flower mites among flowers infected by *Fusarium*. Healthy flowers of the diseased plants contain mites.

Field experiments

To investigate the effects of nectar removal on total nectar production and seed production, we conducted the following field experiments focusing on healthy flowers.

Experiment 1: Response to nectar removal

In January 2002, we randomly selected healthy buds about to open from each of 13 plants (7 healthy and 6 diseased) of *Moussonia deppeana* ($n = 26$ flowers, 14 of healthy plants and 12 from diseased plants) and tagged the buds with plastic rings. The disease status of the plant is not an experimental treatment in this study because plants that become diseased are not a random subset of the entire population. For example, the higher nectar production of diseased plants may not be entirely due to the fungus; it may be that healthy plants with somewhat higher nectar production are also more prone to infection. To measure the effect of nectar removal on total nectar production, mites and hummingbirds were excluded by applying Tanglefoot® (sticky resin; Tanglefoot Co., Grand Rapids, Michigan, USA) to each pedicel of each flower, then flowers were bagged with mosquito-net bags. Undescribed hummingbird flower mites (*Tropicoseius* Baker & Yunker) inhabit *Moussonia* flowers and consume up to 50% of the nectar otherwise available to hummingbirds (Lara & Ornelas, 2001). The reduction in nectar availability can influence hummingbird foraging patterns by increasing the number of probes per flower, and this positively affects *Moussonia* seed production (Lara & Ornelas, 2002). After these exclusions, each flower was subjected to one of the following treatments: (1) 3 removals, one per day for 3 d, during which the flower was in staminate phase for 2 d and pistillate phase for 1 d, or (2) 1 removal during the third day, during which the flower was in pistillate phase. Nectar was extracted carefully during the days after the exclusion with capillary tubes without removing the flowers from the plants. Flowers remained bagged between removals. Note that in this experiment we only examined if *M. deppeana* flowers responded positively to repeated nectar removal (additional nectar replenishment), regardless of their sexual phase. In accordance with the results of a previous pilot study (December 2001 to January 2002), all nectar extractions were made between 1000 and 1700, the hours during which we observed the highest nectar production. Nectar volume per flower was measured using graduated micropipettes (5 µL) and a vernier caliper, sugar concentration was measured (percentage sucrose) with a pocket refractometer (American Optical 10431, Buffalo, New York, USA; range concentration 0–50º BRIX units), and the amount of sugar produced was expressed as milligrams according to Bolten *et al.* (1979).

Experiment 2: Effects of nectar removal on seed production

Buds about to open were chosen randomly from 35 plants (19 healthy plants and 16 diseased plants) in January 2002. Buds were bagged as explained in Experiment 1. To measure the effects of nectar removal on total nectar production and seed production, each flower was subjected to one of the following treatments: (1) 4 removals, once per day for 2 d in which the flower was in staminate phase and 2 d in which the flower was in pistillate phase ($n = 11$ flowers), (2) 2 removals, once per day for 2 d in which the flower was in staminate phase ($n = 9$ flowers), (3) 2 removals, once per day for 2 d in which the flower was in pistillate phase ($n = 10$ flowers), (4) controls for treatments 1, 2, and 3 with nectar being returned to the flower after extraction (10 flowers with 4 removals and returns, 8 flowers with 2 removals and returns during the staminate phase, and 9 flowers with 2 removals and returns during the pistillate phase), and (5) a control with flowers exposed 4 d to natural levels of nectar removal and pollination ($n = 16$ flowers). The natural unbagged treatment acts as a "pseudocontrol" for seed production, although the mechanisms affecting seed production in this treatment are unclear and may be due to resource allocation, pollen load, or both. Note that every treatment had a control in which nectar was removed, then returned. Flowers subjected to repeated removals (with or without nectar returns) were manually cross-pollinated during the second day of pistillate phase by smearing the anther from the donor onto the receptive virgin stigma with pollen grains directly from anthers from randomly selected plant donors. Fruit capsules from experimental flowers were collected *ca* 3 months later (April). Individual plants received a unique treatment or control, and randomly selected flowers from each individual plant represent the sampling unit. Given the abundant seeds per fruit in this species, we used fruit weight as an estimate of female reproductive success (seed production), as Lara and Ornelas (2001) have shown that total seed number increases linearly with fruit weight.

Experiment 3: Effects of nectar removal and micropipette on seed production

In January 2003, we randomly selected buds about to open from 34 plants (18 healthy and 16 diseased) and tagged the buds with plastic rings. To measure the effect of nectar removal on total nectar production and seed production, mites and hummingbirds were excluded from the buds, as described in Experiment 1. After these

exclusions, we applied the following treatment levels: (1) 8 removals, twice every day for 4 d in which the flower was 2 d in staminate phase and 2 d in pistillate phase ($n = 20$ flowers), (2) 1 removal in which the flower was in the second day of pistillate phase ($n = 25$ flowers), (3) a control for multiple insertions with plugged micropipettes, twice every day for 4 d as in treatment 1 (8 insertions total, $n = 9$ flowers), and (4) a control with flowers exposed 4 d to natural levels of nectar removal and pollination ($n = 21$ flowers). The treatment in which plugged micropipettes are inserted into the flower but no nectar is removed acts as a control for the effects of pipette insertion into the flower, including how such insertion might affect nectar and seed production (see Ordano & Ornelas, 2005). A random plant donor was selected to pollinate flowers as described in Experiment 2, and fruit capsules were collected in April to estimate seed production.

STATISTICAL ANALYSES

Total volume production and total sugar production were correlated variables ($r_s = 0.89$, $P < 0.0001$). Therefore, we first did a split-plot completely randomized MANOVA to analyze variation in nectar secretion among flowers with 1 or 3 removals, from both healthy and diseased plants. Using a MANOVA followed by univariate ANOVAs reduces the probability of inflating the Type I error rate. In the model, disease status was the whole plot, removal treatment was treated as the split-plot, and plant was the replication.

The effects of nectar removal on total nectar production (volume) and fruit weight were analyzed using ANOVAs. In the model, removal treatment and disease status were treated as fixed factors, plant was a blocking factor, and nectar volume or fruit weight were the dependent variables. Sugar concentration measurements were not possible for flowers to which nectar was returned, flowers inserted with plugged micropipettes, and those exposed to natural levels of nectar removal. Therefore, the effects of nectar removal on subsequent sugar production were assessed with one-way ANOVAs. We performed Shapiro–Wilk tests to meet parametric analysis assumptions. Nectar volume, amount of sugar, and fruit weight were $\log_{10}$ transformed ($x + 1$), but untransformed data are reported in figures. All statistical analyses were done using Statview and SuperANOVA (Abacus Concepts, Berkeley, California, USA).

Results

EXPERIMENT 1: RESPONSE TO NECTAR REMOVAL

Three removals significantly increased total nectar volume in *Moussonia* uninfected flowers (mean ± SE, 3 removals = 22.69 ± 4.34 μL·flower^{-1}; 1 removal = 10.67 ± 1.91 μL·flower^{-1}, $n = 13$ for each treatment). The MANOVA results indicate a significantly altered nectar profile (Wilks' Lambda = 0.37, $F_{2, 10} = 8.64$, $P = 0.007$), and the disease status, plant (block), and treatment × disease status interaction were not significant ($P > 0.1$). When univariate ANOVAs were performed, the amount of nectar significantly increased with removal intensity ($F_{1, 11} = 6.28$, $P = 0.029$), but the amount of sugar did not (3 removals = 3.44 ± 0.79 mg·flower^{-1}; 1 removal = 2.44 ± 0.42 mg·flower^{-1}, $n = 13$ for each treatment; $F_{1, 11} = 0.39$, $P = 0.54$).

EXPERIMENT 2: EFFECTS OF NECTAR REMOVAL ON SEED PRODUCTION

Nectar removal had a significant effect on total nectar volume production (nested ANOVA; treatment effect, $F_{5, 15} = 17.75$, $P < 0.0001$). Flowers subjected to 4 removals with or without returns produced significantly more nectar than other nectar removal treatments (Figure 1). Visual inspection of data indicates that nectar return consistently decreased nectar production, suggesting that some nectar was lost during manipulations; however, these differences were not statistically significant (Games–Howell procedure, $P > 0.05$). Uninfected flowers from diseased plants produced significantly more nectar (mean ± SE = 21.8 ± 2.56 μL·flower^{-1}, $n = 29$ flowers) than those from healthy plants (16.3 ± 2.27μL·flower^{-1}, $n = 28$ flowers; $F_{1, 15} = 24.29$, $P = 0.0002$). Although the removal treatment × disease status interaction was significant ($F_{5, 15} = 3.73$, $P = 0.0214$), differences between groups of plants were due to flowers on diseased plants with 4 removals and returns producing more nectar than those on healthy plants (Games–Howell procedure, $P > 0.05$). The blocking factor (plant nested within disease status) also had a significant effect on total volume production ($F_{30, 15} = 9.21$, $P < 0.0001$). Total sugar production varied significantly with removal intensity (one-way ANOVA, $F_{2, 17} = 6.41$, $P = 0.0084$), increasing with number of removals (mean ± SE; 4 removals = 6.11 ± 0.68 mg sugar·flower^{-1}, $n = 6$ flowers; 2 removals during staminate phase = 3.05 ± 0.38, $n = 6$ flowers; 2 removals during pistillate phase = 3.53 ± 0.68, $n = 8$ flowers).

Increased nectar production did not appear to affect seed production in *Moussonia* flowers ($F_{6, 25} = 1.12$, $P = 0.3783$; Figure 2). The disease status of plants had a marginal effect ($F_{1, 25} = 3.98$, $P = 0.0568$), and the treat-

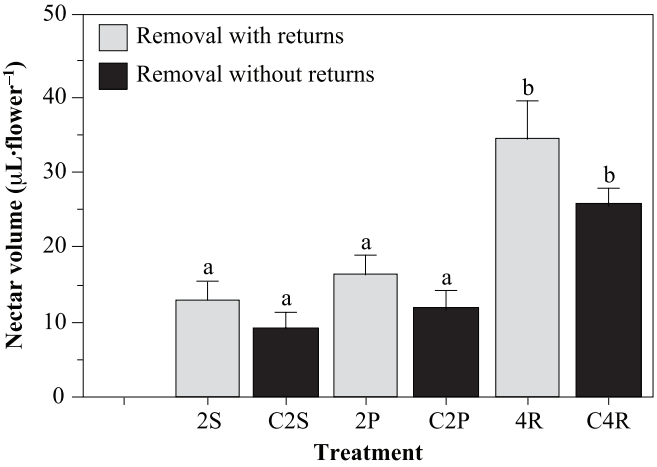

FIGURE 1. The effect of nectar removal on total nectar production (mean + SE) in healthy flowers from both healthy and diseased plants subjected to the following nectar removal treatments: (1) 2 removals, once per day during the staminate phase (2S); (2) a control with nectar being returned to the flower after 2 removals during the staminate phase (C2S); (3) 2 removals, once per day during the pistillate phase (2P); (4) a control with nectar being returned to the flower after 2 removals during the pistillate phase (C2P); (5) 4 removals, once per day from staminate to pistillate phase (4R); and (6) a control with nectar being returned to the flower after 4 removals (C4R). All flowers were bagged and remained bagged between removals. Data with same letters are not significantly different between groups.

ment × disease status interaction was not significant ($F_{6, 25} = 0.86$, $P = 0.5308$). Individual plants (nested within disease status) were significantly heterogeneous for fruit weight ($F_{34, 25} = 2.84$, $P = 0.0041$). Flowers exposed to natural levels of nectar removal and pollination produced fruits as heavy as those exposed to experimental conditions (Games–Howell procedure, $P > 0.05$).

Experiment 3: Effects of nectar removal and micropipette on seed production

Nectar removal had a significant effect on total nectar production in *Moussonia* flowers (Table I). Flowers emptied 8 times produced significantly more nectar (mean ± SE = 25.82 ± 3.22 µL·flower^{-1}, $n = 20$ flowers) than those emptied once (nectar volume = 6.15 ± 0.70 µL·flower^{-1}, $n = 25$ flowers). These differences were independent of disease status, and the removal treatment × disease status interaction was not significant. The blocking factor (plant nested within disease status) had a significant effect on total nectar production. Again, uninfected flowers from diseased plants produced more nectar (mean ± SE = 16.7 ± 3.13 µL·flower^{-1}, $n = 22$ flowers) than those from healthy plants (13.2 ± 2.76 µL·flower^{-1}, $n = 23$ flowers). However, these differences were marginally significant. Total sugar production increased with repeated removal (mean ± SE; 8 removals = 3.29 ± 0.46 mg·flower^{-1}, $n = 20$ flowers; 1 removal during second day of pistillate phase = 1.19 ± 0.14 mg·flower^{-1}, $n = 23$ flowers; one-way ANOVA, $F_{1, 41} = 22.19$, $P = 0.0001$).

Nectar removal had no significant effect on *Moussonia* seed production (treatment effects, $F_{3, 17} = 0.47$, $P = 0.7033$). On average, flowers subjected to repeated removal produced as many seeds as those subjected to 1 removal, 8 insertions with plugged micropipettes, or those exposed to natural levels of pollination (Games–Howell procedure, $P > 0.05$; Figure 3). Disease status did not contribute to explaining variation in fruit weight ($F_{1, 17} = 0.79$, $P = 0.3858$), and the nectar removal treatment × disease status interaction ($F_{3, 17} = 0.47$, $P = 0.7097$) and the block-

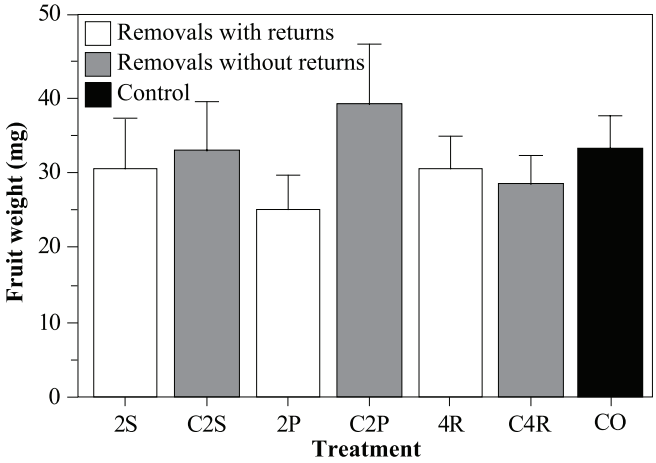

Figure 2. The effect of nectar removal on seed production (mean + SE) in healthy flowers from both healthy and diseased plants subjected to the following nectar removal treatments: (1) 2 removals, once per day during the staminate phase (2S); (2) a control with nectar being returned to the flower after 2 removals during the staminate phase (C2S); (3) 2 removals, once per day during the pistillate phase (2P); (4) a control with nectar being returned to the flower after 2 removals during the pistillate phase (C2P); (5) 4 removals, once per day from staminate to pistillate phase (4R); (6) a control with nectar being returned to the flower after 4 removals (C4R); and (7) a control with flowers exposed to natural levels of nectar removal and pollination (CO). All flowers were bagged and remained bagged between removals. Flowers subjected to repeated removals (with or without nectar returns) were manually cross-pollinated (day 4) with pollen from a single randomly selected individual except for the unbagged flowers exposed to natural levels of pollination.

Table I. Summary of nested blocking ANOVAs on the effect of nectar removal (8 removals *versus* 1 removal) and disease status (diseased plants *versus* healthy plants) on total nectar production in *Moussonia deppeana* uninfected flowers.

Source	df	Sum of of squares	Mean square	F	P
Microlitres of nectar					
Treatment	1	3.0214	3.0214	77.59	0.0001
Disease status	1	0.1417	0.1417	3.64	0.0745
Treatment × disease status	1	0.0107	0.0107	0.28	0.6065
Plant (disease status)	25	3.3358	0.1334	3.42	0.0067
Residual	16	0.6229	0.0389		
Milligrams of sugar					
Treatment	1	0.6951	0.6951	27.41	0.0001
Disease status	1	0.0156	0.0156	0.62	0.4439
Treatment × disease status	1	0.0058	0.0058	0.23	0.6367
Plant (disease status)	23	0.903	0.0393	1.54	0.1855
Residual	16	0.4056	0.0253		

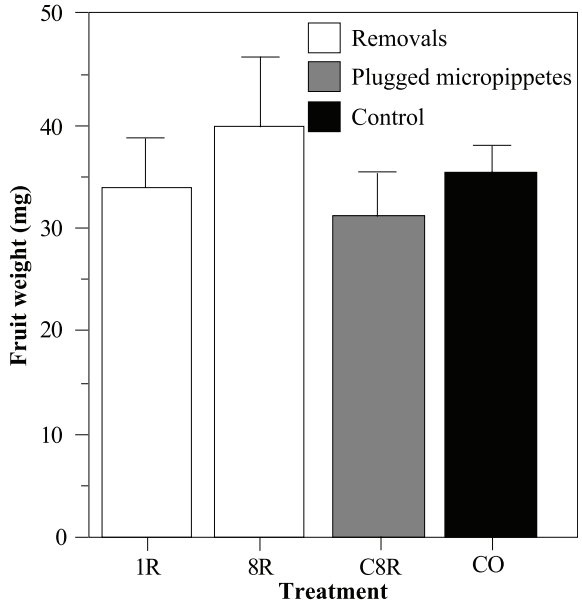

Figure 3. The effect of nectar removal on seed production (mean ± SE) in healthy flowers from both healthy and diseased plants subjected to the following nectar removal treatments: (1) 1 removal, the second day of the pistillate phase (1R); (2) 8 removals, twice every day from the staminate phase to the second day of the pistillate phase (8R); (3) 8 insertions with plugged micropipettes, twice every day from the staminate phase to the second day of the pistillate phase (C8R); and (4) a control with flowers exposed to natural levels of nectar removal and pollination (CO). All flowers were bagged and remained bagged between removals. Flowers subjected to repeated removals (or insertions with micropipettes) were manually cross-pollinated (day 4) with pollen from a single randomly selected individual except for the unbagged flowers exposed to natural levels of pollination.

ing factor (plant nested within disease status) were also not significant ($F_{24, 17} = 1.18$, $P = 0.3614$).

Discussion

POSITIVE RESPONSE TO NECTAR REMOVAL

Our results demonstrate that *Moussonia* flowers replenish removed nectar. The cumulative nectar volume production by flowers with the highest removal intensity (8 removals) was about 4 (4.23) times as high as that by those with the lowest removal intensity (1 removal). This is a particularly strong result: previous experiments involving stimulation of nectar production by nectar removal have yielded values ranging from 1.47 to 3.92 times the control nectar volume (reviewed by Ordano & Ornelas, 2004). By removing nectar, we stimulated replenishment of sugar as well: the amount of sugar increased over twofold (2.74) with increased removal intensity (see also Castellanos, Wilson & Thomson, 2002; Ordano & Ornelas, 2004). However, the nectar removal experiments in our study differed in magnitude of perturbation and duration of experimental manipulation, and these sources of variation may have influenced our results. Experiments were performed by applying 1 to 8 removals, and the duration of experimental manipulation spanned from 1 to 4 d. In all cases and both years, nectar removal yielded positive effects and values ranging from 2.1 to 2.7 times the control nectar volume and from 1.4 to 2 times the control amount of sugar. Although some animal-pollinated plant species respond negatively to nectar removal, in other plant species the effect is positive (see Ordano & Ornelas, 2004 for additional references). Our results are consistent with an exploratory meta-analysis conducted by Ordano and Ornelas (2004) regarding the application of nectar removal manipulation among species. Their meta-analysis revealed 3 general patterns: (1) the magnitude of the response was not influenced by the magnitude of perturbation (number of removals) or flower longevity as a measure of duration of experimental manipulation, (2) the magnitude of the response were low for nectar volume, but stronger responses to nectar removal were detected for species inhabiting wet tropical habitats, and (3) there were small negative effects for amount of sugar. Their meta-analysis showed that plant species are conservative in their expenditure of sugar and the additional production of nectar after nectar removal.

Although *Moussonia* flowers had the highest rate of nectar replenishment reported to date (higher magnitude of the response according to the meta-analysis by Ordano & Ornelas, 2004; see Figure 5 in Ordano & Ornelas, 2004), it remains unclear whether a flower's response to nectar removal relates to selection pressures imposed by floral visitors, both mutualists and antagonists (*e.g.,* Lara & Ornelas, 2001; Castellanos, Wilson & Thomson, 2002; Lara & Ornelas, 2003; Ordano & Ornelas, 2005). Also, it is likely that plants inhabiting wet tropical forests have more water, enabling them to replenish removed nectar. In environments with more limited resources, it is likely that the magnitude and direction of the response to nectar removal is determined by limits on (1) the average amount of energy that a flower can offer in response to repeated removal and/or (2) the availability of energy, water, amount of sugar, or other nectar constituents for the plant. The treatments used in our study increased the amount of nectar produced in only a few of the *ca* 25 flowers (Lara & Ornelas, 2003) on a plant, assuming that nectar production in one flower was independent of nectar production in other flowers of the same plant. A strength of our study is that the nectar removal treatments included an experimental set-up and a number of controls that help rule out alternative explanations for the results at the flower level. We found that the magnitude of the response (nectar replenishment) was influenced by the magnitude of the perturbation (number of removals) but not by the sexual phase of the flower or damage by micropipette insertions. No costs of nectar replenishment were detected in terms of seed production, but detection of such costs might depend strongly on the level at which reproduction is analyzed because resource allocation pathways are different at the branch, individual plant, fruit, and seed levels (Obeso, 2004). Only experiments in which all flowers on a plant have been subjected to nectar treatment might detect such costs.

NECTAR REMOVAL EFFECTS ON SEED PRODUCTION

This study assessed how nectar replenishment affects seed production by experimentally removing nectar from manually cross-pollinated flowers. We found that additional nectar production in *Moussonia* flowers subjected to the same removal treatment had no effect in terms of seed production. Previous studies have shown that the expenses of nectar production can range from costly to beneficial (see Ordano & Ornelas, 2005 for additional references). The potential cost of nectar production after repeated removals was not expressed as a reduction in seed production possibly because (1) hummingbirds were less attracted to experimental plants and treated the unbagged control flowers differently compared to those from non-manipulated plants, (2) nectar production in *Moussonia* is a very small part of the energy budget (see also Harder & Barrett, 1992), (3) the number of pollen grains deposited naturally or experimentally on stigmas likely varied among treatments, and, therefore, pollen loads were not enough to fertilize most ovules, and/or (4) resource allocation pathways during reproduction are regulated at the plant level. Although our study was not designed to distinguish among these non-mutually exclusive explanations, the number of seeds produced by each flower in *M. deppeana* depends on the number of visits and number of probes by the pollinator, increasing with pollinator visitation (Lara & Ornelas, 2001; 2002). Also, we used flowers from a single plant as pollen donors to minimize possible genetic effects and to simplify our experimental design. It is likely that the number and origin (single *versus* mixed genotypes) of pollen grains deposited naturally and experimentally on the stigma play an important role in determining seed set. The fact that plants had bagged inflorescences may have affected the behaviour of hummingbirds visiting the plants. However, all birds encountered the same experimental conditions. Lastly, our pollination and removal methodology ignored the whole-plant context of plant mating, in particular, the actual fitness consequences of high replenishment rates in individual flowers that depend on pollen export and import from experimentally affected flowers, but also the

effects on the overall attractiveness of the entire plant and on hummingbird movements among flowers on the plant. Because the plant has been shown to be self-compatible (Lara & Ornelas, 2001; T. Velázquez & J. F. Ornelas, unpub. data), geitonogamous crosses (within-plant pollen transfer) and xenogamous crosses (between-plant pollen transfer) are confounded in our study. By having flowers exposed to natural pollination (geitonogamy and xenogamy), possible differences in seed production might have been reduced compared with manual cross-pollinated flowers. Currently, we have no data to discuss further the likelihood of these suggested explanations; however, we believe that the potential benefits of nectar replenishment rates (maximizing pollen movement while keeping low geitonogamy rates) may rule out potential costs in terms of seed production. Clearly, our data reveal a need for more studies on the effects of nectar replenishment in terms of both male and female fitness, particularly in self-compatible protandrous species. Given that flower-level effects of nectar replenishment on seed production were not detected in *M. deppeana*, our results are consistent with the idea that nectar production is a cheap process in terms of female reproductive success. However, our manipulations were carried out at the flower level, and resource limitation may act at the plant level. As tradeoffs between nectar production and seed production could be expressed at the plant level and/or as long-term effects, the costs of nectar replenishment need further study.

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