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HUMMINGBIRDS AS VECTORS OF FUNGAL SPORES IN *MOUSSONIA DEPPEANA* (GESNERIACEAE): TAKING ADVANTAGE OF A MUTUALISM?[1]

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Hummingbirds act as vectors of *Fusarium moniliforme* spores on protandrous flowers of *Moussonia deppeana*. The resulting interactions between the pathogen and plant–pollinator interactions were investigated in a 4-yr study to determine the pathogen's impact on host flowering phenology, flower longevity, nectar production, and fruit and seed production. We also evaluated hummingbird behavior on healthy and diseased plants and its effectiveness on spore transmission. Individual plants expressed the disease from year to year, and new infected individuals were detected every year. A fraction of the flowers in a plant expressed the disease, and this varied among and within years. Diseased plants produced more inflorescences, buds, and open healthy flowers than did healthy plants. Further, diseased plants bore proportionally fewer pistillate flowers than did healthy plants when considering only healthy flowers. Neither nectar nor fruit production differed between healthy and diseased plants, but healthy plants produced more seeds. Infected flowers were retained longer than uninfected ones, producing an additional 2 mg $\cdot$ μL^{-1} $\cdot$ $flower^{-1}$ of nectar sugar. Hummingbirds visited more flowers on diseased plants than they did on healthy plants, regardless of number and sexual phase. Most pollen and spores were deposited within plants. These behavioral outcomes may promote geitonogamy and limit fungal spore mixing.

Key words: anther-smut disease; flower longevity; flowering phenology; Gesneriaceae; *Fusarium moniliforme*; *Moussonia deppeana*; nectar production; pollinator behavior; protandry; seed production; spore deposition.

The production of pseudoflowers (Roy, 1993; Pfunder and Roy, 2000), floral scents (Batra, 1991; Patt, 1992), and colors (Batra and Batra, 1985; Roy, 1993) induced by fungi to attract pollinators and the transmission of spores to the plants that they visit (Roy, 1993, and references therein) suggest that some fungi exploit the mutualistic relationships between flowering plants and their pollinators. Studies have shown that some rusts, smuts, and other pathogenic fungi can use pollinators to ferry gametes between different fungal individuals as a way to promote sexual reproduction and/or use their host's pollinators to transfer infectious spores to a new host (Jennersten, 1983; Batra and Batra, 1985; Alexander and Maltby, 1990; Batra, 1991; Roy, 1996).

The most studied anther smut, *Ustilago violacea* Pers (= *Microbotryum violaceum* Pers) (Basidiomycetes: Ustilaginales), affects members of the Caryophyllaceae (Baker, 1947; Alexander, 1989; Biere and Honders, 1996; Shykoff, Bucheli, and Kaltz, 1997). It has been documented that these fungi can alter the host flowering phenology, floral morphology, and flower visitation as a way to ensure spore transmission (Lee, 1981; Biere and Honders, 1996; Shykoff and Kaltz, 1998). Although the epidemiology of the *Ustilago*–Caryophyllaceae systems is well known, pollinator transmission of fungal spores is known for other groups of fungi (Batra and Batra, 1985; Burdon, Ericson, and Müller, 1995; Ericson, Burdon, and Müller, 1999; Pfunder and Roy, 2000). Insect transmission is important for these fungal diseases (Jennersten, 1983, 1988; Bultman and White, 1988; Batra, 1991), however vertebrate-pollinated plants are also at risk.

We have discovered that *Fusarium moniliforme* (Scheld.) Snyder & Ansen (Deuteromycota: section *Liseola*) infects flowers of *Moussonia deppeana* (Schlecht. & Cham.) Hanst. (Gesneriaceae), a protandrous, hummingbird-pollinated perennial shrub. Here, we describe (1) disease prevalence and intensity of infection, (2) effects on the host flowering phenology, flower morphology and longevity, and nectar and seed production, and (3) hummingbird visitation patterns and their influence on spore and disease transmission. To our knowledge, this is the first study that has attempted to evaluate hummingbirds as vectors of plant diseases.

MATERIALS AND METHODS

Study site—Fieldwork was conducted from November to March 1998–2002, in a small *Moussonia* population (~300 plants) growing in a remnant of cloud forest (29 ha), in the Parque Ecológico Francisco Xavier Clavijero near Xalapa, Veracruz, Mexico (19°30′ N, 96°57′ W; at 1225 m above sea level).

Study system—*The pathogen*—*Fusarium moniliforme* (hereafter *Fusarium*) is a fungal pathogen characterized by its microconidia occurring in chains (Booth, 1971). The aerial mycelium is generally dense with a powdery appearance. *Fusarium* is widespread in both humid and subhumid temperate zones and extends into subtropical and tropical zones throughout the world (Booth, 1971). Host plant families include Asclepiadeaceae, Bromeliaceae, Caryophyllaceae, Colvolvulaceae, Euphorbiaceae, Moraceae, Orchidaceae, Polemoniaceae, Rosaceae, and Rubiaceae (Booth, 1971). It is a major crop

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pathogen of several Gramineae in which it may cause seedling blight, scorch, foot rot, stunting, and hypertrophy (Booth, 1971). Wind and rain dispersal of *Fusarium* has been confirmed in cornfields (Ooka and Kommedahl, 1977; Pascoe, 1990), and European corn borer (Lepidoptera, Pyrallidae) larvae also disseminate spore among leaves causing kernel rot and symptomless infection of maize kernels (Sobek and Munkvold, 1999). However, little is known about how *Fusarium* spores are disseminated in the wild.

The host plant—Moussonia deppeana (hereafter *Moussonia*) is an abundant, 1–3-m-tall perennial subshrub distributed in shaded areas of forests from southern Mexico to Honduras (Wiehler, 1982). This species flowers from November to mid-March. The axillary inflorescences have pronounced peduncles with compound cymes of four flowers each (Wiehler, 1975). On average, eight flowers are open on healthy plants at mid-flowering season (mean ± 1 SD, 8.01 ± 3.5, $N = 20$; Lara and Ornelas, 2001). The orange-red tubular flowers are solely pollinated by the amethyst-throated hummingbird (*Lampornis amethystinus*) (Lara and Ornelas, 2001, 2002). We have not observed other animals visiting the flowers. *Moussonia* flowers are protandrous, opening in the morning and staying open 4 d (Lara and Ornelas, 2002). Each flower passes through a 2-d male period (staminate phase), followed by a 2-d female phase (pistillate phase) (Lara and Ornelas, 2001). More nectar is secreted on average during the staminate phase (1.98 ± 1.6 μL/flower, $N = 20$) than in the pistillate phase (1.12 ± 0.5 μL/flower, $N = 20$). Sugar concentration (BRIX scale) is the same, on average, between flower phases (16% of sucrose; C. Lara, unpublished data).

Fusarium grows endophytically and asymptomatically within host plant tissues and sporulates in the anthers. In our study site, diseased plants commonly produce both infected and uninfected flowers. Undescribed hummingbird flower mites (*Tropicoseius* Baker & Yunker) inhabit *Moussonia* flowers (Lara and Ornelas, 2001), but we have not observed them among flowers infected by *Fusarium*. Healthy flowers of the diseased plants contain mites.

The vector—Lampornis amethystinus Swainson (Trochilidae) (hereafter *Lampornis*) is a traplining hummingbird, endemic to the highlands of Central Mexico and Middle America. In our study site, *Lampornis* hummingbirds are residents and feed from flowers of *Palicourea padifolia* (Rubiaceae), *Lobelia laxiflora* (Lobeliaceae), and bromeliads (Contreras and Ornelas, 1999; Lara and Ornelas, 2001). During winter, these hummingbirds pollinate and mostly rely on *Moussonia* flowers for nectar.

***Field procedures**—Prevalence and intensity of Fusarium infection*—In November 1998, 72 plants were randomly selected prior to flowering along the main trail of our study site. Plants were monitored monthly for 3 yr to determine (1) what fraction of flowers are infected per plant, (2) whether the overall level of infection varies among years, and (3) whether individual plants were consistently infected from year to year. We evaluated disease prevalence as the number of plants that produced infected flowers relative to the 72 randomly selected plants and disease intensity as the proportion of infected flowers relative to the total number of open flowers in each diseased plant. The measure of disease prevalence includes healthy plants (i.e., plants with 0% infected flowers).

Association between disease status and plant traits—In December 1999, five diseased flowering plants were randomly selected to determine the effect of *Fusarium* on flower longevity. We chose six infected buds about to open on each plant. These were identified by the dark-white color of the young anthers. Three buds were bagged with bridal netting and the other three remained unbagged to determine whether flower longevity among infected flowers is influenced by floral visitors. We followed both groups of flowers until wilting. Longevity of uninfected flowers on healthy plants was estimated at the same time, as previously reported (Lara and Ornelas, 2001, 2002).

Flowering phenology was followed in detail from November 2000 to March 2001. We randomly selected 33 healthy and 11 diseased plants from the 72 above. For each one, we recorded monthly the number of inflorescences, buds, and open flowers and the number of staminate or pistillate flowers out of the total number of open flowers. We discriminated between uninfected and infected flowers within diseased plants. We also measured plant size (height in centimeters) and flower size (corolla length in millimeters; flower entrance width in millimeters) of both types of plants. In March 2001, we counted and then harvested all fruit capsules produced by healthy ($N = 11$) and diseased ($N = 11$) plants in the 2000–2001 flowering season (standing crop). Given that seed number increases linearly with fruit mass in this species (Lara and Ornelas, 2001, 2002), we used total fruit mass as an estimate of seed production and female reproductive success.

Nectar production—Twenty flowering plants were randomly selected (10 healthy and 10 diseased) to determine the effect of *Fusarium* on *Moussonia* nectar production. From each plant, we chose three buds ready to open and bagged them with bridal netting to exclude floral visitors. Flower buds chosen for sampling of nectar production in diseased plants were infected. Flower mites were excluded by applying Tanglefoot (sticky resin; Tanglefoot Co., Grand Rapids, Michigan, USA) as they may consume up to half the nectar otherwise available to pollinators (Lara and Ornelas, 2001). Nectar was extracted the following day at 0800 with capillary tubes without removing the flowers from the plants. Nectar volume (in microliters) was measured using graduated micropipettes (5 μL) and a ruler (Kearns and Inouye, 1993). Nectar secretion pattern was determined for infected and uninfected flowers over their lifetime. Data were taken once every morning (0800) from the same set of flowers, allowing the nectar to accumulate for 24 h between measurements.

Flowers subjected to repeated nectar removals might be stimulated to produce additional nectar and these then might confound real secretion patterns (Castellanos, Wilson, and Thomson, 2002, and references therein). To explore this possible confounding factor, we conducted additional nectar measurements on different healthy and diseased plants. Three sets of buds were chosen and bagged to exclude floral visitors to let nectar accumulate for 12, 15, and 18 h. The following day, the amount of accumulated nectar was collected only once on 1-d flowers and measured at 0800, 1100, and 1300.

Nectar standing crop—Because pollinators are probably responding to nectar standing crop, we also extracted the nectar available in flowers that had been exposed to floral visitors and measured its volume, concentration, and mass of sugar. Data were collected from individual infected ($N = 43$) and uninfected staminate ($N = 70$) and pistillate ($N = 54$) flowers between 0800 and 1300. Nectar samples from 0800 ($N = 61$), 1100 ($N = 52$), and 1300 ($N = 54$) were taken to evaluate eventual variation in sugar through the peak of hummingbird activity (Lara and Ornelas, 2002). Nectar volume was measured as described above and sugar concentration (percentage sucrose) with a pocket refractometer (American Optical 10431, Buffalo, New York, USA; range concentration 0°–50°, BRIX units). The amount of sugar produced was expressed as milligrams after Kearns and Inouye (1993).

Pollinator behavior—Twenty flowering plants were randomly selected to determine whether hummingbirds discriminate between healthy ($N = 10$) and diseased plants ($N = 10$). Behavioral observations were conducted on each individual plant (one plant per day) in November and December 1999 from 0800 to 1200 (hummingbird activity peaks during this period; Lara and Ornelas, 2002). We recorded (1) number of visits to each focal plant, (2) number of flowers visited per foraging bout, and (3) number of probes to each flower visited. After observation, we recorded the number and sexual phase of open flowers on each focal plant. Plants were observed on separate days as a way to increase the likelihood of flower visitation by different hummingbird individuals. By observing only one plant per day, the treatment (diseased vs. healthy) is confounded by date. Paired observations may have been preferable but impractical under field conditions.

Effectiveness of hummingbirds on spore and disease transmission—We used fluorescent dye as a pollen analogue and direct observations of hummingbird behavior to track pollen and spore movements. In November 1998, pollinators were visually followed and the distance between flower visits estimated, as described by Parra, Vargas, and Eguiarte (1993). Behavioral observations were made for 5 h/d during five consecutive days, covering different areas every day that contained 10–20 healthy and diseased plants. In

December 1998, fluorescent powder dyes (USR Optonix, Hacketstown, New Jersey, USA) were applied to freshly opened 1-d infected flowers located at the center of a patch containing healthy ($N = 22$) and diseased plants ($N = 11$). Fluorescent dye was applied early in the morning before pollinators became active (as described by Waser, 1988). Flowers were marked on a focal plant with one dye color. Anthers were dusted with dye using a cotton swab. The following day at noon, we collected all open flowers within a 10-m radius of the focal plant and recorded the distances between the source and recipient plants. We confirmed the presence of dyes in flowers with a dissecting microscope.

In January 2000, an inoculation experiment was conducted to evaluate the effectiveness of *Lampornis* in the transmission of *Fusarium* spores. Ten healthy flowering plants, each with three buds, were randomly selected ($N = 30$ flowers) and bagged with bridal netting. Hummingbird inoculation was then conducted on 1-d flowers (staminate; $N = 15$ flowers) and 3-d flowers (pistillate; $N = 15$ flowers) by using a stuffed *Lampornis* hummingbird as spore vector. Inoculations were accomplished by probing the hummingbird's bill three times into the corolla of a donor flower with infected anthers and then once into the corolla of a recipient uninfected flower. We mimicked hummingbird behavior in flowers as closely as possible (Lara and Ornelas, 2002). Spore loads carried by the stuffed bird are likely different from those carried by live birds because spores from a specific infected flower (donor) may persist on hummingbirds for many flowers, mixing with pollen grains. Instead, we only performed inoculations with spores to simplify our experimental design. Only one plant was used as spore donor to minimize possible confounding factors due to host plant. Most spores adhered to the hummingbird's forehead. As much as possible, we removed the residual spores with a paintbrush prior to performing the next inoculation. After inoculations were performed, staminate and pistillate flowers remained bagged and were daily checked for symptoms of infection until they wilted (flower retention).

Statistical analyses—We used a 2 (infection or not, year 1) × 2 (infection or not, year 2) × 2 (infection or not, year 3) contingency table to determine whether individual plants were consistently infected from year to year. Variation in intensity of infection over time was analyzed using one-way repeated-measures ANOVA. In the model, year was treated as a fixed factor and the monthly intensity of infection was the repeated measure.

Flower production among plants with and without infection over time was analyzed using repeated-measures ANOVAs. In the model, disease status of the plant (healthy vs. diseased) was treated as a fixed effect; the number of inflorescences, buds, and open flowers and the proportion of staminate and pistillate flowers per plant over the flowering period (5 mo) were the repeated measures.

Nectar production among plants with and without infection over time was also evaluated with repeated-measures ANOVA. In the model, disease status of the flower (infected vs. uninfected) was treated as a fixed factor and daily nectar production (4 d) as the repeated measure. Between-plant variation was assessed with disease status as the main factor and plants nested within disease status. Along with the main effects, flower age × disease status, and flower age × plant(disease status) interactions were included in the model. Variation in accumulated nectar and nectar standing crop between groups of plants as a function of time for the nectar to accumulate was assessed with a two-way ANOVA.

Plant and flower size, nectar production over the lifetime, and fruit standing crop among diseased and healthy plants were assessed using one-way ANOVAs. Variation in the number of seeds (fruit mass) among healthy and diseased plants was contrasted with a two-way ANOVA. Between-plant variation was assessed with disease status as the main factor and plant nested within disease status.

Lastly, pollinator behavior on healthy and diseased plants was assessed with ANCOVAs (Zar, 1984), as plants varied in the number of open flowers and proportion of staminate and pistillate flowers over the observation period. In the model, disease status was a fixed factor, number of open flowers per plant and proportions of each flower phase were the covariates, and number of flowers visited per bout and number of probes per flower were the response variables. Because we observed plants on different days (between-plant variation), the plant factor was not included in our model.

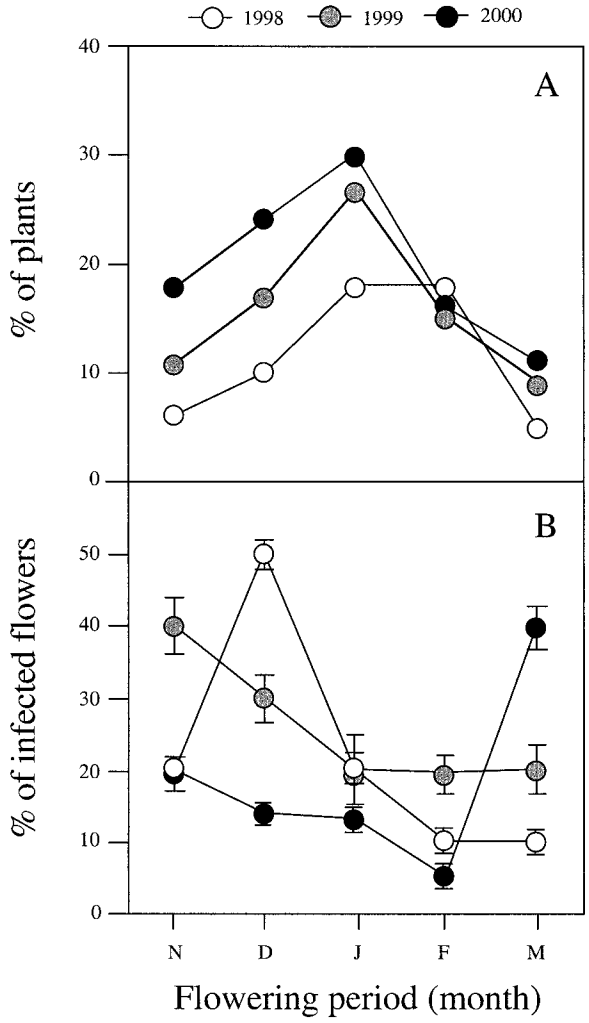

Fig. 1. (A) Disease prevalence (in percentages) and (B) intensity of infection (means ± 1 SE) by *Fusarium moniliforme* on 72 *Moussonia deppeana* plants over a 3-yr study. Note that prevalence decreases over the season as some plants cease to show symptoms of infection (infected flowers) (see Results). Intensity of infection was measured only for plants bearing some infected flowers.

All statistical analyses were done using General Linear Modeling with StatView and SuperANOVA (Abacus Concepts, 1989, 1996). Data were arcsine, square-root, or $\log_{10}$ transformed as needed before analysis to correct for nonnormality or heteroscedasticity, but untransformed data are reported in text and figures.

RESULTS

Prevalence and intensity of Fusarium infection—The proportion of infected individuals increased from 22% in 1998 to 30% in 2000. Disease prevalence increased over the three years of the study and within each year between November and January (18–30%; Fig. 1A), but then decreased. Diseased plants continued to flower but produced only healthy flowers, i.e., the same individuals continued to flower but their effective status changed because they bore only healthy flowers for the later censuses. New infected individuals were detected every year (see below). The probability that a plant was infected

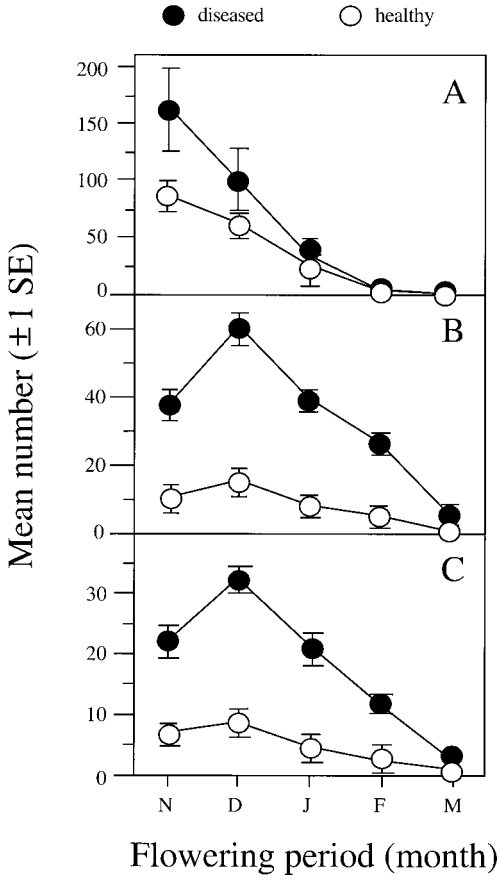

Fig. 2. Variation in (A) number of buds, (B) number of open flowers, and (C) number of inflorescences per plant among diseased and healthy plants of hummingbird-pollinated *Moussonia deppeana* in the 2000–2001 flowering season.

was not independent of year ($\chi^2 = 32.8$, df = 3, $P < 0.001$); all plants infected in year 1 were also infected in year 2 and year 3. The percentage of infected flowers on diseased plants (infection intensity) averaged 24.1 ± 1.0% (range 1–70%) and varied among years (means ± 1 SD; 1998, 21.9 ± 15.8, N = 15 plants; 1999, 25.9 ± 16.8, N = 16; 2000, 18.4 ± 14.5, N = 21). Infection intensity generally decreased over the season, with the exception of March 2000, when the eight flowering infected plants produced numerous infected flowers (Fig. 1B). Results of the repeated-measures ANOVA indicated that the intensity of infection differed over the three years of the study (year effect, $F_{2,196} = 9.13$, $P < 0.001$) and varied within each year (month effect, $F_{4,196} = 45.76$, $P < 0.001$). The significant year × month interaction ($F_{8,196} = 37.32$, $P < 0.001$) was due to the dramatic infection increase in December–January 1998 and March 2000.

Association between disease status and plant traits—Diseased plants were not significantly taller than healthy plants (diseased, 1.34 ± 0.14 m, N = 25; healthy, 1.52 ± 0.11 m, N = 33; one-way ANOVA, $F_{1,56} = 1.99$, $P = 0.16$), but produced more inflorescences, buds, and open healthy flowers than did healthy plants (Fig. 2, Table 1). The number of inflorescences and open flowers per inflorescence peaked in December and the numbers decreased as the flowering season progressed (Fig. 2). The proportion of the healthy pistillate flowers on diseased plants was lower than on healthy plants, though the proportion of healthy staminate flowers did not differ between healthy and diseased plants (Fig. 3, Table 1).

Fungal infection on *Moussonia* resulted in the transformation of flowers into spore-producing structures; stamens developed with anthers producing conidia and ascospores. Infected flowers were all sterile, no pollen was produced during the staminate phase, and the subsequent pistillate phase was inhibited. Flowers infected with *Fusarium* were easily identified even when anthers were not yet sporulating. Their dark-white color contrasts with the yellow in healthy flowers. We confirmed the presence of spores (and the absence of pollen grains) on infected flowers with a dissecting microscope. Infected and uninfected flowers were similar in size (corolla length: infected, 25.01 ± 0.3 mm, N = 40; uninfected, 24.93 ± 0.2 mm, N = 50; one-way ANOVA, $F_{1,88} = 0.02$, P = 0.88; flower entrance width: infected, 6.13 ± 0.1 mm, N = 40; uninfected, 6.16 ± 0.1 mm, N = 50; one-way ANOVA, $F_{1,88} = 0.07$, $P = 0.79$), except for the dark-white color of anthers among the infected flowers. No differences in flower longevity were observed between bagged and unbagged infected flowers. However, both groups of flowers were retained invariably two more days before wilting (N = 30) than were healthy flowers. Uninfected flowers lasted only 4 d even when they were bagged.

Fruit standing crop was similar between healthy (27.5 ± 1.2, N = 33) and diseased plants (25.5 ± 1.1, N = 11) ($F_{1,42}$ = 0.63, $P = 0.43$). However, healthy plants produced significantly heavier fruits (more seeds) (0.217 ± 0.002 g, N = 11) than diseased plants (0.195 ± 0.002 g, N = 11) (disease status effect, $F_{1,534} = 42.77$, $P < 0.001$). Individual plants varied in fruit mass [plant(disease status) effect, $F_{20,534} = 2.16$, $P <$ 0.01].

TABLE 1. Results of repeated-measures ANOVAs examining variation in the number of inflorescences, buds, and open flowers, and the proportion of staminate flowers and pistillate flowers among plants with and without *Fusarium moniliforme*. Disease status (healthy vs. diseased plants) was treated as a fixed effect, and all the flowering phenology variables were the repeated measures over the 5 mo of the flowering period. Flower phase was evaluated only for 4 mo. Numbers were square-root transformed and proportions were arcsine transformed. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$.

	Between-plant variation					Within-plant variation							
	Disease status			Error		Month			Month × disease status			Error	
Variable	df	MS	*F*	df	MS	df	MS	*F*	df	MS	*F*	df	MS
No. of buds	1	84.91	6.68*	42	12.71	4	534.81	98.34***	4	17.40	3.20*	168	5.43
No. of flowers	1	381.86	116.66***	42	3.27	4	69.66	54.90***	4	9.15	7.12***	168	1.27
No. of inflorescences	1	175.97	134.78***	42	1.31	4	38.26	64.75***	4	6.14	10.39***	168	0.59
Proportion of staminate	1	0.04	0.67 NS	23	0.06	3	0.06	1.37 NS	3	0.02	0.28 NS	69	0.05
Proportion of pistillate	1	0.63	10.85**	23	0.06	3	0.07	1.15 NS	3	0.01	0.13 NS	69	0.06

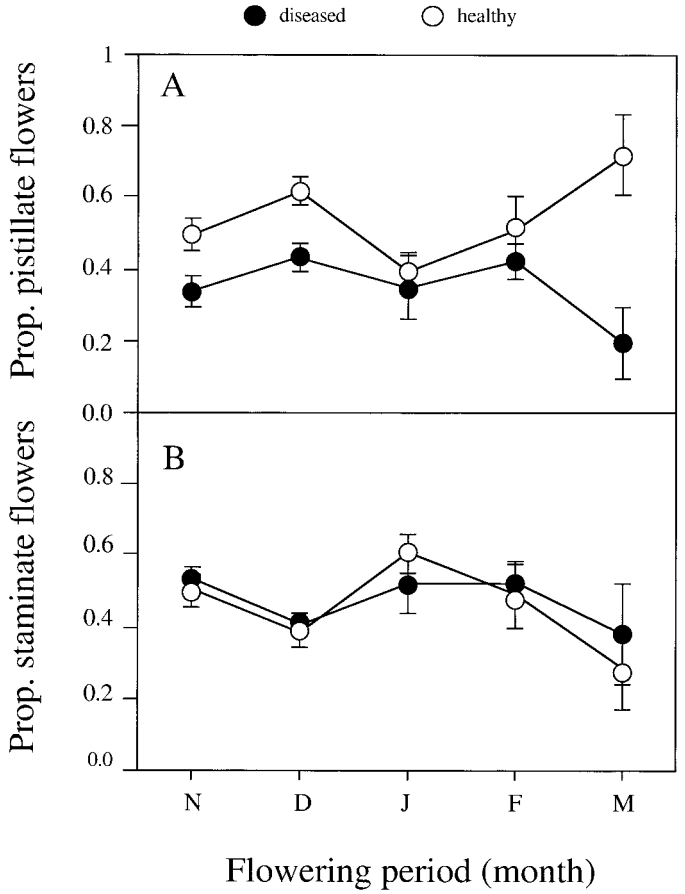

Fig. 3. Proportion of (A) healthy pistillate and (B) healthy staminate flowers of *Moussonia deppeana* over time on healthy plants and plants diseased by *Fusarium moniliforme* in the 2000–2001 flowering season. Data are means ± 1 SE.

Nectar production—Nectar production during the first 4 d did not differ between infected and uninfected flowers (repeated-measures ANOVA; disease status effect, $F_{1,120} = 0.63$, $P = 0.43$). Nectar production increased over time (flower age effect, $F_{3,120} = 183.04$, $P < 0.001$; Fig. 4) similarly for diseased and healthy flowers (flower age × disease status interaction, $F_{3,120} = 0.77$, $P = 0.51$). Plants varied for nectar production [plant(disease status) effect, $F_{18,120} = 2.01$, $P = 0.03$] and individual plants varied in how their nectar production changed with flower age [flower age × plant(disease status) interaction, $F_{54,120} = 1.79$, $P < 0.01$]. Total nectar volume produced by infected flowers over their lifetimes (6 d) (39.95 ± 0.11 μL/flower, $N = 30$) was significantly higher than the total nectar produced by uninfected flowers over shorter life spans (4 d) (26.88 ± 0.09 μL/flower, $N = 30$; t test = 90.7, df = 58, $P < 0.001$).

On average, infected and uninfected flowers accumulated the same nectar volume (means ± 1 SE; infected, 1.64 ± 0.22 μL/flower, $N = 46$; uninfected, 1.36 ± 0.19, $N = 45$; disease status, $F_{1,70} = 2.45$, $P = 0.12$). Although flowers accumulated nectar over time (time-of-day effect, $F_{2,70} = 5.05$, $P < 0.01$), rate of nectar accumulation was independent of disease status (disease status × time-of-day interaction, $F_{2,70} = 1.27$, $P = 0.29$).

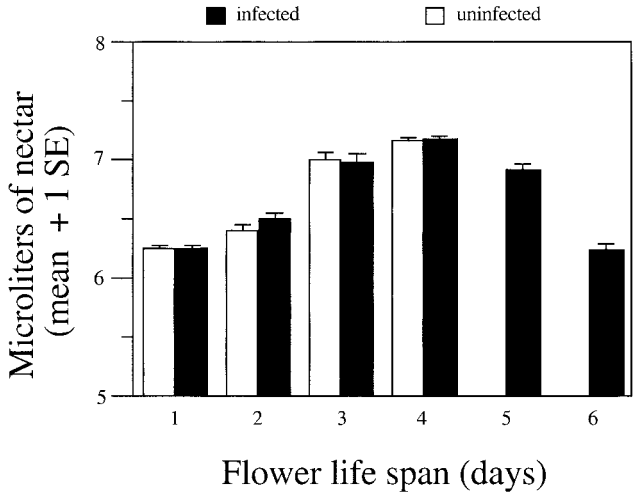

Fig. 4. Nectar production by infected and uninfected flowers of *Moussonia deppeana* over their lifetimes. Data were taken in the 2000–2001 flowering season.

Nectar standing crop—The amount of nectar available to floral visitors did not differ with disease status or time of day (disease status effect, $F_{2,158} = 0.72$, $P = 0.48$; time-of-day effect, $F_{2,158} = 0.34$, $P = 0.71$; disease status × time-of-day interaction, $F_{4,158} = 0.44$, $P = 0.77$). Infected flowers had less sugar-concentrated nectar (14.9 ± 0.5 BRIX, $N = 29$) than uninfected flowers (staminate, 16.5 ± 0.5, $N = 40$; pistillate, 16.2 ± 0.4, $N = 32$), but these differences were marginally significant ($F_{2,92} = 2.64$, $P = 0.076$) and were consistent over time (disease status × time-of-day interaction, $F_{4,92} = 1.98$, $P = 0.10$). On the other hand, sugar concentration varied almost significantly over time, decreasing over the course of the day (time-of-day effect, $F_{2,92} = 3.06$, $P = 0.051$). Lastly, there were no significant differences between infected and uninfected flowers in the total number of milligrams of sugar per volume over time (disease status effect, $F_{2,92} = 0.07$, $P = 0.93$; time-of-day effect, $F_{2,92} = 0.26$, $P = 0.77$; disease status × time-of-day interaction, $F_{4,92} = 0.76$, $P = 0.55$).

Pollinator behavior—Neither the number of open flowers nor the proportion of pistillate and staminate flowers affected pollinator behavior on *Moussonia* individual plants. Because the F values for the disease status × covariates interactions were not significant (results not shown), we removed them from the ANCOVAs. After removing such effects from the ANCOVAs, the F value for disease status was significant only for the number of flowers visited per bout (Table 2).

Hummingbirds visited more flowers per foraging bout on plants with infected flowers (6.1 ± 0.4, $N = 30$) than they did on healthy plants (2.6 ± 0.3, $N = 18$), independently of flower number and proportion of each flower phase on a given plant (Table 2). Probing behavior did not differ between healthy and diseased plants (diseased, 1.8 ± 0.2 probes/flower, $N = 30$; healthy, 1.7 ± 0.2 probes/flower, $N = 18$; Table 2).

Hummingbird visitation rate (number of visits a plant received during the observation period) was significantly higher among diseased plants (3.0 ± 0.2, $N = 10$) than healthy plants (1.9 ± 0.3, $N = 10$; t test = 2.8, $P = 0.011$). However, on a per flower basis, visitation rates were similar among flowers regardless of the plant disease status (means ± 1 SE; flowers of diseased plants, 0.5 ± 3.9 visits · flower^{-1} · bout^{-1}, $N =$

TABLE 2. Results of ANCOVAs examining the relationship between pollinator behavioral responses and (A) number of open flowers, (B) number of staminate flowers, and (C) number of pistillate flowers among healthy and diseased plants of *Moussonia deppeana*, after removing the nonsignificant interactions between disease status and covariates. Data were square-root or arcsine transformed before analysis. ***$P < 0.0001$.

	No. flowers visited/bout			No. probes/flower		
Covariates	df	MS	*F*	df	MS	*F*
A) Open flowers						
Disease status	1	4.81	22.57***	1	0.01	0.06
Number/plant	1	0.18	0.83	1	0.01	0.02
Error	45	0.21		45	0.10	
B) Staminate flowers						
Disease status	1	7.05	34.43***	1	0.03	0.29
Number/total	1	0.57	2.77	1	0.15	1.56
Error	45	0.20		45	0.10	
C) Pistillate flowers						
Disease status	1	4.75	21.98***	1	0.11	1.03
Number/total	1	0.05	0.23	1	0.14	1.43
Error	45	0.22		45	0.10	

30; flowers of healthy plants, 0.4 ± 6.0 visits · flower^{-1} · bout^{-1}, $N = 18$; t test $= -1.58$, $P = 0.12$).

Effectiveness of hummingbirds on spore and disease transmission—Pollen dispersal distances were limited despite the typical traplining behavior of the pollinator. The average flight distances between flowers by *Lampornis* was 143.7 ± 40.8 cm (mean ± 1 SE) ($N = 89$), with 90% of the visits within less than 100 cm (Fig. 5A). The distance of dye transfers from the dye source to recipient plant ranged from 0 to 100 cm (Fig. 5B). The mean distance (± 1 SE) of dye transfer was 237.2 ± 65.8 cm with a median of 90.5 cm. The longest distance was 8 m, but 80% of the collected flowers with fluorescent particles were also recovered between 0 and 100 cm from the focal infected flower. The distributions for dye transfer distance and pollinator flight distance were leptokurtic. Both estimates suggest that most pollen (or spores) removed from an infected flower can be deposited on the next visited flower.

The inoculation experiment showed differences in flower retention between 1-d and 3-d flowers contaminated with fungal spores. All 15 1-d staminate flowers were retained exactly for 6 d, but the transition to pistillate phase did not occur. By contrast, all 15 3-d pistillate flowers wilted 1 d after inoculation. Because disease symptoms only appear the next season, we could not determine whether these inoculations led to infection or not.

DISCUSSION

The use and abuse of plant–hummingbird mutualisms by Fusarium—It has been suggested that some plant pathogenic fungi could alter some phenological aspects of their hosts, as a way to achieve greater conspicuousness and attraction of pollinators (Roy, 1994). We found that in *Moussonia* plants infected by *Fusarium*, infected flowers were retained longer than uninfected ones, both in the presence and absence of hummingbird visitation, as has been observed for other species of pollinator-transmitted fungal diseases (Baker, 1947; Alexander, 1990; Shykoff, 1997). Such extended floral life spans of infected flowers may enhance fungal transmission. Also,

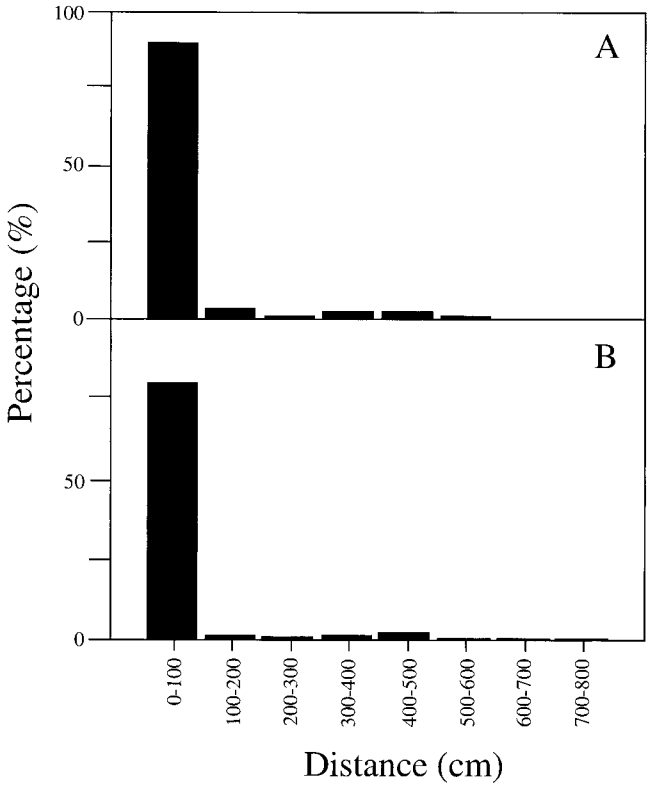

Fig. 5. Spore transmission measured as (A) pollinator flight distance between inflorescences and (B) distances between dye donor and recipient.

diseased plants produced more flowers overall, and more healthy flowers, than do healthy ones. It is not clear if this is a consequence of phenotypic manipulation by the fungus to attract vectors (Alexander, 1989; Elmqvist et al., 1993) or whether plants with high flower production or higher attractiveness to vectors were more likely to become infected (Shykoff, Bucheli, and Kaltz, 1997). This latter process could lead to pathogen-mediated selection on flower production, and we can test for this by comparing flower production and other floral traits of healthy plants that subsequently became infected over our study with those that did not. Unfortunately, flower production data are not yet available for plants from the season before they became infected, so additional observations are necessary to distinguish between these two processes.

Despite the larger, healthy floral display of diseased plants fruit crops were similar in size, though they contained fewer seeds. It is possible that the large floral display led to more geitonogamy (Harder and Barrett, 1995) and loss of seeds through inbreeding depression. The systemic fungal infection could also have direct negative effects on seed production. In addition, spore deposition on healthy flowers can reduce seed set (Alexander, 1987; Carlsson-Granér et al., 1998; Marr, 1998) in part by impeding pollen germination (Marr, 1998). We also found that spore deposition alters phenological pattern, preventing the normal transition from staminate to pistillate flowers. Indeed, on diseased plants healthy pistillate flowers were underrepresented, compared with their healthy counterparts in the population. Local spore deposition within plants could have led to this relative lack of pistillate flowers.

Flower life span should evolve in the presence of pollinator-borne diseases, with high disease pressure reducing optimal

life span of healthy flowers to reduce their risk of contracting disease (Shykoff, Bucheli, and Kaltz, 1996). Optimal mating strategies of male and female flowers in the presence of sexually transmitted disease may not necessarily be those that minimize the risk of infection and disease spread in a population (Guldbrandtsen, 1997; Thrall, Antonovics, and Bever, 1997; Kaltz and Shykoff, 2001). Male-biased infection rates have been documented for dioecious *Silene alba* (Caryophyllaceae) (Alexander, 1989; Biere and Antonovics, 1996; Biere and Honders, 1996; Carlsson et al., 1990), possibly because male plants are selected to be more promiscuous. To our knowledge, there is virtually no information on how disease risk would operate in dichogamous, protandrous plants. The increased flower retention by 1-d experimentally inoculated flowers excluded from pollinators implies that the extended flower retention is in reaction to fungal contamination. For the fungus it may be advantageous to extend flower retention by a few days if the infection process requires some time. By contrast, pistillate flowers inoculated with *Fusarium* spores were not retained longer. Plastic responses in flower longevity to pollination events, particularly to pollen removal, have been documented for protandrous plants (Devlin and Stephenson, 1985; Richardson and Stephenson, 1989), but whether *Fusarium* is taking advantage of this plasticity in *Moussonia* staminate flowers remains to be investigated. Experiments of removal and deposition of pollen combined with spore inoculation should help to discern whether the increase in flower longevity is a response to pollen removal and/or fungal contamination.

A reduction in nectar production among plants diseased by fungi has been reported for *Viscaria vulgaris* (Jennersten, 1988) and *Silene latifolia* (Shykoff and Bucheli, 1995), and it has been suggested that this reduction in attractiveness can lower insect visitation (Alexander, 1990; Jennersten and Kwak, 1991; Shykoff and Bucheli, 1995) and influence disease transmission. We found no difference in nectar production between infected and uninfected flowers of *Moussonia* during the first 4 d of their lifetimes nor nectar produced or available on a daily basis (standing crop and accumulated nectar data). However, infected flowers produced nectar over a longer period of time (2 mg/μL of additional sugar). This additional nectar secretion may entail an energy cost to diseased plants (Pyke, 1991; M. Ordano and J. F. Ornelas, Instituto de Ecología, AC, unpublished data) and could explain in part their observed reduction in seed production. Alternatively, lower seed set in diseased plants could be due to more pollen limitation in diseased than healthy plants. That is, pollen losses to infected flowers in diseased plants may reduce pollen deposition on uninfected flowers. In addition to the costs of added flowers, flower retention, and sugar production, it may be that there is an energy cost to being infected (i.e., the infection is systemic), not necessarily related to reproductive traits.

Hummingbirds as vectors of fungal spores—We believe that the deposition of *Fusarium* spores on flowers is primarily due to the action of hummingbirds. We acknowledge the limitation of our study in terms of assuming that hummingbirds are the only or most important disease vectors. Here, we have experimentally demonstrated successful spore deposition through hummingbird inoculation, and pollen, and presumably, spore deposition mainly occurred to the nearest neighboring flowers. Similar results of pollen deposition have been documented for several hummingbird-pollinated plants (Waser, 1982; Wolf and Hainsworth, 1990; Parra, Vargas, and Eguiarte, 1993). Although our two estimates of pollen (and spore) deposition were similar, the fluorescent dyes reached the longest distance. This was expected, as the flight distance method considers the unrealistic supposition of no pollen carryover (Levin and Kerster, 1974). Thus, the fluorescent dye method may be a more realistic estimate (Marr et al., 2000; but see Waser, 1988). Fluorescent dye was moved by hummingbirds primarily within plants and between plants in close proximity to each other (80% of the dye transfers occurred within an 8-m radius of the source plant). The consequences of this behavioral outcome were not evaluated in our study; however, flights within *Moussonia* individuals should promote geitonogamy and limit fungal spore mixing if we assume that only a single fungal strain infects a plant.

Hummingbirds visited more flowers on diseased than on healthy plants. This is not surprising, because diseased plants were more conspicuous, bearing 7–9 times as many combined healthy and infected flowers, and pollinators are known to respond to display size. However, on a per flower basis, hummingbird visitation rates did not differ between diseased and healthy plants (Schmid-Hempel and Speiser, 1988). Nor was flower number a good predictor of visitation rate within the healthy or the infected groups, as indicated by our ANCOVA results. Our hummingbird behavioral observations and distances of dye deposition suggest that much pollen and spore transmission will occur within rather than between plants. Indeed, the larger number of open flowers and longer visitation bouts of hummingbirds on diseased plants suggests that more geitonogamy might occur (Harder and Barrett, 1995). Further, much spore deposition will be expected to occur within a plant because, despite the relatively small number of flowers on a diseased plant that are infected (about 26%), these flowers remain open and provide nectar rewards for longer. Nevertheless, the appearance of new diseased individuals over the years shows that successful long-distance transmission events occur.

In summary, *Fusarium* appears to modify *Moussonia* flowering phenology, increasing allocation of resources to pollinator attraction and reward. Increased floral display enhanced pollinator visitation by hummingbirds, which are effective vectors of *Fusarium* spores, thus increasing the dissemination of this fungal disease. These results suggest that pollinator preference might affect the dynamics of disease transmission and the outcomes of the plant–hummingbird mutualistic interaction.

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