

REPRODUCTIVE ECOLOGY OF DISTYLOUS *PALICOUREA* *PADIFOLIA* (RUBIACEAE) IN A TROPICAL MONTANE CLOUD FOREST. I. HUMMINGBIRDS' EFFECTIVENESS AS POLLEN VECTORS¹

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The adaptiveness of distyly has been typically investigated in terms of its female function, specifically pollen receipt. However, pollen loads on stigmas can only provide moderate support for Darwin's hypothesis of the promotion of legitimate crosses. To determine the effectiveness of hummingbirds as pollen vectors between floral morphs and the consequences in terms of male (pollen transfer) and female function (pollen receipt) in *Palicourea padifolia* (Rubiaceae), floral visitors, their foraging modes, and temporal patterns of floral visitation were observed and documented. Differences in pollen and stigma morphology, pollen flow, rates of pollen deposition, and/or stigmatic pollen loads were then evaluated for their contribution toward differences in reproductive output between floral morphs. A pollination experiment with stuffed hummingbirds that varied in bill size was done to evaluate the contribution of bill variation toward differences between floral morphs in pollen receipt and pollen transfer and female reproductive output. Anthers of long-styled flowers contained significantly more and smaller pollen grains than those of short-styled flowers, independently of corolla and anther lengths. The shape and orientation of the stigma lobes differed between morphs and were significantly longer among short-styled flowers. Hummingbird visitation rates did not differ significantly between floral morphs, and foraging movements from focal plants towards neighboring plants were independent of floral morph. Stigmatic pollen loads under field conditions and those after controlled hummingbird visitation, along with rates of pollen accumulation through the day indicated that stigmas of short-styled flowers receive proportionately more legitimate (intermorph) pollen grains than did those of long-styled flowers. However, the species of hummingbird was marginally significant in explaining variation in pollen deposition on stigmas. Lastly, intermorph pollinations of *P. padifolia* resulted in significant differences in fruit production between floral morphs, independent of pollination treatment and pollinator species; short-styled flowers proportionately developed almost twice the number of fruits developed by long-styled flowers.

Key words: distyly; hummingbird; *Palicourea padifolia*; pollen transfer; pollinator behavior; Rubiaceae; stigmatic pollen loads.

Distyly is a balanced polymorphism in which about half of the plants in a population have long styles and short stamens (hereafter L-morph), whereas the remainder possess short styles and long stamens (hereafter S-morph) (Barrett, 1992). Characteristically, the two morphological groups also form mating groups, with each morph able to set seed only when pollinated by the other morph (Stone, 1995). Although this balanced polymorphism is designed for reciprocal pollen transfer (Barrett, 1990), the efficacy of such a mechanism often depends on pollinator effectiveness (Beach and Bawa, 1980). If pollen flow is asymmetrical, then differential reproductive success between floral morphs can appear (Ganders, 1974).

Darwin (1877) hypothesized that the reciprocal placement of stamens and styles between floral morphs is a mechanical device to promote animal-mediated cross-pollination among

the morphs (legitimate pollination). According to Darwin's hypothesis, pollen from different stamen levels would adhere to various parts of the animal's body corresponding to the position where compatible stigmas would contact the animal. Several workers have investigated the magnitude of legitimate pollination in populations of heterostylous plants by using the marked size heteromorphism of pollen produced by different floral morphs, specifically the number of legitimate (compatible) and illegitimate (incompatible) pollen grains deposited on stigmas (stigmatic pollen loads) presumably by the legitimate pollinators (Ganders, 1979; Barrett and Glover, 1985; Stone, 1995; Ree, 1997). Other workers have examined the location of pollen grains of either morph on pollinators, typically insects, captured while foraging on heterostylous species (Ganders, 1979; Barrett and Wolfe, 1986; Wolfe and Barrett, 1989).

The aim of our study was to determine the effectiveness of hummingbirds as pollen vectors between floral morphs of *Palicourea padifolia* (Rubiaceae) and the consequences of their morphology and behavior in terms of fruit production. We first evaluated whether differences in pollen size and number, rates of pollen deposition, and stigmatic pollen loads account for the observed differences between floral morphs in reproductive output. We then observed the behavior of floral visitors to describe the assemblage composition and foraging modes and to determine temporal patterns of floral visitation. Lastly, we conducted a pollination experiment with stuffed hummingbirds representing bill size variation in the known hummingbird community of *P. padifolia* to evaluate its contribution in

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female reproductive fitness between floral morphs. In addition, we present evidence of differences in pollen and stigma morphology to explain the proximal causes of gender specialization in *P. padifolia*.

MATERIALS AND METHODS

Study site—Fieldwork was done from March to October 2002 in the Parque Ecológico Francisco Xavier Clavijero near Xalapa, Veracruz, Mexico ($19^{\circ}30' N$, $96^{\circ}57' W$; at 1225 m above sea level). A full description of the area is given by Contreras and Ornelas (1999).

Study species—*Palicourea padifolia* (Roem. & Schult.) C.M. Taylor and Lorence (Rubiaceae) is an abundant shrub (2–7 m in height) in disturbed areas of cloud forests from southern Mexico to Panama (Taylor, 1989). It can be recognized by reddish-purple pyramidal, terminal inflorescences with yellow, 1-d flowers in cymose clusters (Taylor, 1989).

Our study population is morphologically distylous (mean \pm 1 SD, style height, L-morph = 15.9 ± 1.2 mm, S-morph = 8.4 ± 1.1 mm) (Contreras and Ornelas, 1999). The L-morph flowers have smaller stamens and corollas (stamen length = 12.1 ± 0.99 mm; corolla length = 14.1 ± 1.35 mm) than S-morph flowers (stamen length = 15.0 ± 1.06 mm; corolla length = 16.9 ± 1.05 mm; Contreras and Ornelas, 1999). Stigma and anther heights are not exactly reciprocal (stigma–anther separation: L-morph = 4.8 ± 0.97 mm; S-morph = 6.2 ± 1.13 mm; Contreras and Ornelas, 1999), although the area of presentation is reciprocal. Anthers are lower in L-morph (anther height = 11.5 ± 1.08 mm) than in S-morph flowers (15.6 ± 1.03 mm; Contreras and Ornelas, 1999), but similar in appearance. Flowering time at Parque Clavijero extends from March until August, and maximum flower availability is reached between May and mid-June (Contreras and Ornelas, 1999). Both floral morphs produce, on average, the same number of inflorescences per plant, initiate the same number of flower buds per inflorescence, and open the same number of flowers per inflorescence (Contreras and Ornelas, 1999). Of the 11 hummingbird species, bees, and butterflies that visit flowers of *P. padifolia*, territorial hummingbirds are likely the most effective pollinators (Contreras and Ornelas, 1999).

Fruits are fleshy drupes and significantly larger among L-morph plants (mean \pm 1 SE; fruit mass, L-morph = 0.461 ± 0.01 g, $N = 160$, S-morph = 0.44 ± 0.01 g, $N = 150$; fruit length, L-morph = 8.45 ± 0.07 mm, $N = 160$, S-morph = 8.21 ± 0.08 , $N = 150$; C. González and L. Jiménez, unpublished data), whose color changes from green when immature to purplish-black at ripening (Ree, 1997; Contreras and Ornelas, 1999). Fruits have typically two seeds; seeds are significantly larger in S-morph plants (mean \pm 1 SE; seed mass, L-morph = 0.019 ± 0.001 g, $N = 320$, S-morph = 0.023 ± 0.002 g, $N = 300$; seed length, L-morph = 4.07 ± 0.036 mm, $N = 320$, S-morph = 4.19 ± 0.033 mm, $N = 300$; C. González and L. Jiménez, unpublished data). Fruit development usually starts in May and asynchronous ripening extends typically until October (Contreras and Ornelas, 1999). On average, fruit standing crops are similar between floral morphs (Contreras and Ornelas, 1999; Ornelas et al., 2004), but fruit maturation is higher in S-morph plants (C. González, J. F. Ornelas, and L. Jiménez, unpublished data). In our study site, fruits are consumed by the Social Flycatcher (*Myiozetetes similis*), Grey Catbird (*Dumetella carolinensis*), Common Bush-Tanager (*Chlorospingus ophthalmicus*), and Black-headed Saltator (*Saltator atriceps*) (Contreras and Ornelas, 1999; A. Hernández, M. Ordano, and E. Peresbarbosa, unpublished data), but their role as seed dispersers is unknown.

Pollen and stigma morphology—We collected several flowers of each morph from the field and preserved them in ethanol (70%). At least 24 h later, individual stigmas, anthers, and pollen grains stained with Lugol were observed and photographed (4 \times , 10 \times , 20 \times) under a dissecting microscope (Nikon Eclipse E600, Tokyo, Japan) to document morphological differences between floral morphs. Samples were fixed in a 9 : 1 : 1 ethanol : formalin : acetic acid solution (FAA) as collected and 24-h later gradually dehydrated (50, 60, 70, 80, 90, and 100%). Dehydrated samples were critical-point-dried with liquid CO₂, then coated with palladium gold, observed, and photographed

using a scanning electron microscope (JEOL JSM-5600LV, München, Germany).

Pollen grains were counted on one anther per flower to compare stigmatic pollen loads between those observed under field conditions and those after controlled hummingbird visitation (see below: Pollen donation capabilities of hummingbirds). In June 2002, we measured the length of one anther from each of two flowers of 30 plants of each morph. Corolla length (in millimeters) of each of the flowers was also measured with a digital caliper. Ripe anthers were stained with aniline blue and squashed into drops of alcohol to count the number of pollen grains per anther directly with a compound microscope (10 \times) (Zeiss, Oberkochen, Germany) and to measure (diameter, in micrometers) 10 pollen grains per sample with a micrometer.

Stigmatic pollen loads—In the field, the proportions of pollen present on stigmas of each floral morph were estimated to determine whether pollen flow was disassortative (Ree, 1997). If pollen flow was random, stigmas should receive L-morph and S-morph pollen in the same proportion as the relative frequencies of each pollen type in the population. Because anther removal after anthesis was impractical under field conditions, particularly in L-morph flowers that were damaged by fine forceps, and putative floral changes might modify pollinator visitation (conspicuousness and attractiveness; e.g., Temeles and Rankin, 2000), intact flowers were collected in the afternoon (between 1200 and 1300), placed horizontally in Petri dishes, and carefully transported to the laboratory within 10 min. In the laboratory, flowers were dissected and stigmas removed and mounted on microscope slides in a jelly made of gelatin (3.5 g), glycerol (25 mL), phenic acid (1 g), and purified water (21 mL). Pollen grains were counted with a compound microscope (Zeiss) and a micrometer and classified by morph based on their size (Contreras and Ornelas, 1999).

Pollen deposition rates on stigmas exposed to pollinators for different number of hours were also determined by collecting 20 stigmas of each floral morph at 0900, 1200, and 1500. Pollen grains deposited on stigmas were then counted as described.

Floral visitation—We conducted field observations on 28 focal plants (15 L-morph and 13 S-morph) to describe the assemblage composition and foraging modes of floral visitors and to determine daily patterns of floral visitation. We watched each plant for 2 to 5 h and never returned to that plant during the study. For each plant, we recorded species, time of visitation, number of probes per flower, and the number of flowers visited per foraging bout of each floral visitor. Movements within the focal plant and toward neighboring plants by foraging animals were also recorded. We then followed only foraging hummingbirds that visited flowers on a focal plant and classified them as: (1) flew to another plant of the opposite floral morph, (2) flew to another plant of the same floral morph, (3) flew to a branch and perched there for several minutes until foraging again, or (4) flew out of sight. Observations were undertaken in May over several days in areas where *P. padifolia* was abundant and constituted the dominant food source for foraging nectarivores.

Pollen donation capabilities of hummingbirds—In the laboratory, stuffed hummingbirds varying in bill length were used to estimate their abilities to deposit pollen on virgin stigmas of both floral morphs. Pollen deposition was accomplished by inserting the hummingbird bill into the donor corolla and then into the recipient corolla. This procedure was repeated six times for each of three hummingbird species on both legitimate and illegitimate crosses. True effectiveness of a pollinator is determined by the proportion of pollen grains picked up from one flower and deposited in the next (Rademaker et al., 1997), but using more than one stuffed individual per hummingbird species was not feasible. We used a medium-billed hummingbird that was the most frequent floral visitor of *P. padifolia* in the study area (*Amazilia cyanocephala*; 19.8-mm exposed culmen) and two less frequent floral visitors that represented the extremes in bill size variation (*Eugenes fulgens*, 28-mm exposed culmen; *Atthis heloisa*, 12.3-mm exposed culmen). Pollinated stigmas were then mounted in gelatin on microscope slides. Pollen grains deposited on stigmas were then counted.

Pollination effectiveness of hummingbirds—In July 2002, the influence of variation in hummingbird bill morphology on effectiveness in pollinating *P. padifolia* flowers was evaluated to account for the differences between floral morphs in reproductive output. Thirty flowering plants (13 L-morph and 17 S-morph) were chosen haphazardly across the population, and approximately five inflorescences per plant were bagged with netting. Flowers were then pollinated the following day using stuffed hummingbirds as pollen vectors. The hummingbird's bill was inserted into the donor corolla, then into the corolla of the opposite morph. This procedure was repeated in two treatment levels (one vs. five probes) using (1) a long-billed hummingbird (*E. fulgens*), (2) a medium-billed hummingbird (*A. cyanocephala*), and (5) a short-billed hummingbird (*A. heloisa*) to mimic variation in bill size. We used similar number of replicates per treatment level (i.e., one flower per treatment level in each plant of each floral morph). Two unmanipulated flowers were also chosen from each experimental plant and used as controls: one flower was exposed to floral visitors and the other remained bagged. All treatment levels were applied to each of the plants. Most pollen adhered to the hummingbird's bill and forehead. As much as possible, we removed the residual pollen grains with a paintbrush prior to the next pollination. After pollination, recipient and donor flowers were measured (corolla length in millimeters) with a digital caliper and marked with thin plastic rings. Flowers remained bagged for one day, except those exposed to pollinators (controls), and monitored every 2 wk until fruit production.

Statistical analyses—We analyzed the relationship between pollen production and flower size with ANCOVA (Zar, 1984). In the model, floral morph was a fixed factor, corolla and anther lengths were covariates, and numbers of pollen grains and pollen size were the dependent variables. Variation in stigmatic pollen loads as a function of floral morph was assessed with a nested two-way ANOVA. Between-plant variation was assessed with floral morph as the main factor and with plants nested within floral morph.

Differences in floral visitation between floral morphs were assessed with Mann-Whitney tests. A 2×4 contingency table was used for testing the independence of hummingbird movements and floral morphs. Variation in pollen donation capabilities among hummingbirds as a function of direction of pollen flow was assessed with a two-way ANOVA. The same procedure was used to document the variation in accumulated pollen grains of stigmas between floral morphs as a function of time for the pollen to accumulate under natural conditions.

For the pollination experiment, each experimental flower was categorized based on whether fruits developed after 2 mo of pollination with stuffed hummingbirds. Using a 2×2 contingency table, the proportion of flowers that produced fruits was compared between floral morphs that received the same pollination treatment (one probe, five probes, open to natural pollination). Several flowers and inflorescences were lost during the experiment and were not taken into account. We also used a three-way test of independence between fruit production, pollinator species, and pollination direction (L \rightarrow S and S \rightarrow L). For each pollinator species, floral morph and fruit production were tested with a *G* test of independence with Williams' correction, as recommended for small sample sizes by Sokal and Rohlf (1981). A significant *G* value for pollination with five probes indicates that, within each type of pollinator, the effects of pollen limitation differ in the two floral morphs. A significant *G* value for open pollination implies several potential differences between floral morphs, including differences in hummingbird visitation or fruit rate abortion.

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 non-normality or heteroscedasticity, but untransformed data are reported in text and figures.

RESULTS

Stigma and pollen morphology—Stigmas of both morphs are bilobed. Stigma lobes ranged from 0.66 ± 0.02 mm long ($N = 120$) in L-morph flowers to 1.94 ± 0.03 mm long ($N =$

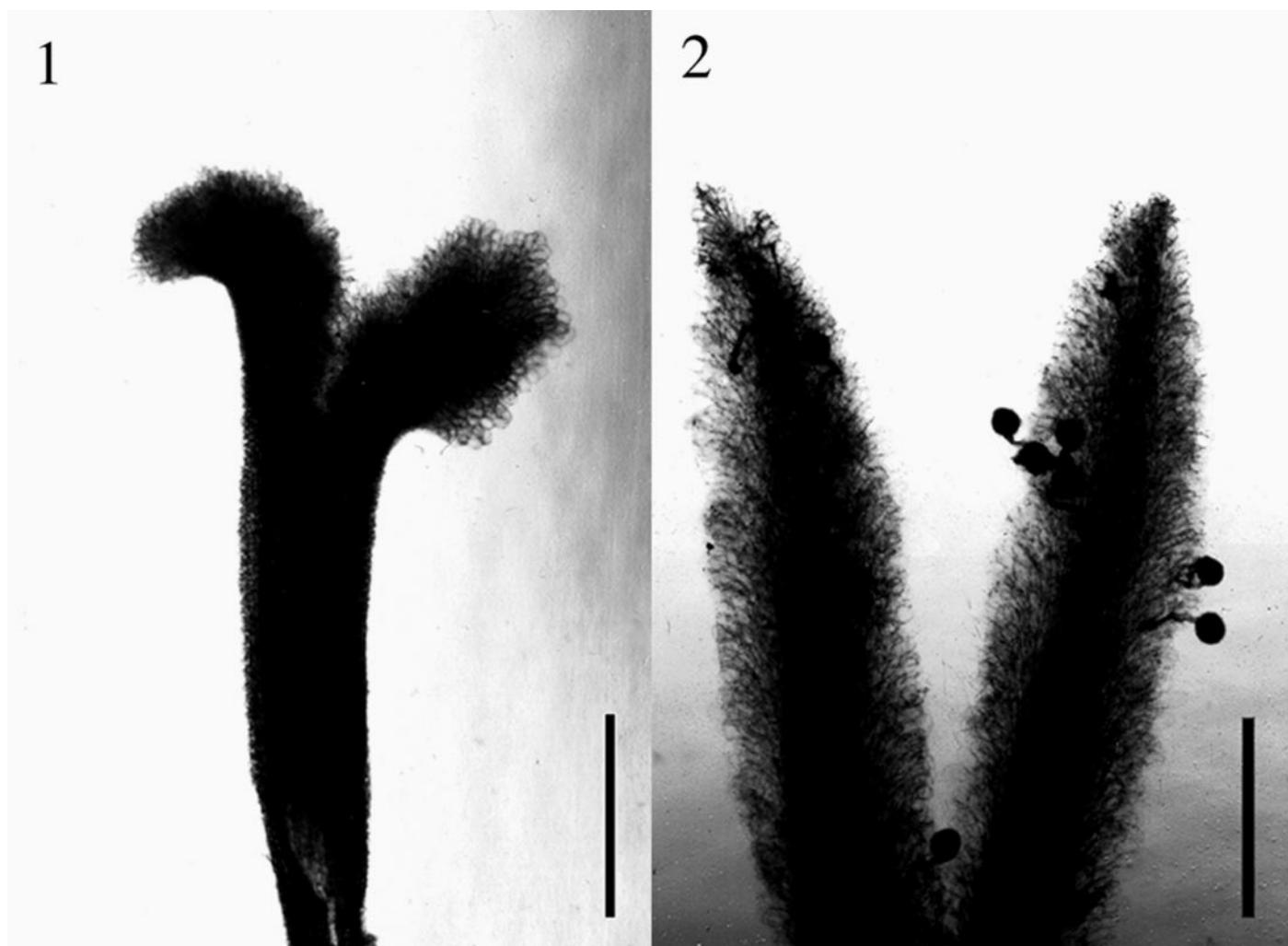
120) in S-morph flowers (one-way ANOVA, $F_{1,238} = 1482.9$, $P = 0.0001$). The shape, length, and the orientation of the stigma lobes vary between morphs (Figs. 1–2). L-morph flowers have wider, more capitate stigmas, whereas those of S-morph flowers are more elongate and lanceolate. As a consequence, the surface of the stigma in S-morph flowers is larger than the surface of stigma in L-morph flowers. The orientation of the stigma lobes ranges from widely spreading and recurved in the L-morph flowers to slightly deflected in S-morph flowers. The interior surface of the stigma and the sinuses between the lobe bases are uniformly papillose, but number, shape, and length of the stigmatic papillae were not quantified.

Anthers of L-morph flowers contained significantly more pollen grains (mean \pm SE; 335.1 ± 10.4 pollen grains per anther) than those of S-morph flowers (267.1 ± 11.2), independently of corolla and anther lengths (Table 1). In contrast, pollen grains were significantly larger in S-morph flowers (mean \pm SE; 99.9 ± 0.6 μm) than those of L-morph flowers (65.4 ± 0.5 μm) and this difference was not independent of corolla length (Table 1). This possible allometric relationship was explored further for each floral morph by regressing pollen diameter with corolla length (log transformed). We found that pollen size significantly increased as corolla size increased only in the L-morph flowers (L-morph, $Y = 1.425 + 0.337X$; $r^2 = 0.22$; S-morph, $Y = 1.976 + 0.019X$; $r^2 = 0.001$).

Stigmatic pollen loads—Mean stigmatic pollen loads under natural conditions differed significantly between floral morphs (ANOVA; floral morph effect; $F_{1,48} = 53.6$, $P = 0.0001$). Within-plant variation was not statistically significant (plant [floral morph] effect; $F_{10,48} = 1.6$, $P = 0.13$). Stigmas of S-morph flowers received more pollen grains (mean \pm SE, 56.7 ± 5.2) than did stigmas of L-morph flowers (17.8 ± 3.2). Based on pollen size, stigmas of S-morph flowers received more legitimate (24.7 ± 3.5) and illegitimate (27.9 ± 2.2) pollen than those of L-morph flowers (legitimate = 2.9 ± 0.5 ; illegitimate = 14.1 ± 2.9), and less than 8% on average were hidden in the preparations and not classified. However, stigmas of S-morph flowers received proportionately more legitimate (intermorph) pollen grains than did those of L-morph flowers (one-way ANOVA, $F_{1,48} = 8.61$, $P = 0.0051$; Fig. 3).

Overall, pollen deposition through time was the same between floral morphs (mean \pm 1 SE, L-morph = 29.4 ± 4.1 pollen grains per stigma, $N = 60$; S-morph = 35.8 ± 4.2 , $N = 60$; floral morph, $F_{1,114} = 2.35$, $P = 0.1278$). Although pollen deposition varied over time (time-of-day effect, $F_{2,114} = 17.07$, $P < 0.0001$), the rate of pollen deposition was not independent of floral morph (floral morph \times time-of-day interaction, $F_{2,114} = 11.53$, $P = 0.0001$; Fig. 4A). Interestingly, stigmas of S-morph flowers received proportionally more legitimate pollen over time (mean \pm SE = 0.67 ± 0.4 , $N = 55$) than did stigmas of L-morph flowers (0.14 ± 0.03 , $N = 51$; two-way ANOVA, $F_{1,100} = 120.99$, $P = 0.0001$), and this morph difference did not change over time (time-of-day effect, $F_{2,100} = 16.97$, $P = 0.0001$) and the floral morph \times time-of-day interaction was also significant ($F_{2,100} = 13.41$, $P = 0.0001$; Fig. 4B).

Floral visitation—A total of 180 visits by hummingbirds (82%) and insects (18%) were registered during our observations (140 h), and plants typically received from 1 to 65 visits (mean \pm 1 SD = 15.3 ± 13.5 visits/plant) in about 5 h of observation. There were no significant differences between flo-



Figs. 1–2. Floral traits of L-morph (long styles, short stamens) and S-morph (short styles, long stamens) flowers of *Palicourea padifolia*. 1. Capitate stigma of L-morph flowers. 2. Elongate stigma of S-morph flowers. Scale bars = 2 mm.

TABLE 1. Results of ANCOVAs for the regression of the mean (A) number of pollen grains and (B) pollen diameter against corolla and anther lengths in L-morph (long styles, short stamens) and S-morph (short styles, long stamens) flowers of *Palicourea padifolia*. Nonsignificant interactions between the floral morph and the covariates were removed from the models.

Source	df	MS	F	P
A) Number of pollen grains				
Floral morph	1	110.85	16.60	0.0001
Corolla length	1	3.42	0.51	0.4753
Residual	117	6.68		
Floral morph	1	87.04	13.00	0.0005
Anther length	1	1.52	0.23	0.6341
Residual	117	6.69		
B) Pollen diameter				
Floral morph	1	0.009	18.36	0.0001
Corolla length	1	0.005	10.74	0.0014
Corolla length (floral morph)	1	0.004	8.55	0.0041
Residual	116	0.000		
Floral morph	1	0.771	1431.81	0.0001
Anther length	1	0.000	0.47	0.4946
Residual	117	0.001		

ral morphs in the total number of visits to a given plant (L-morph = 6.2 ± 3.5 visits, $N = 15$ plants; S-morph = 6.7 ± 4.9 visits, $N = 13$ plants; $U = 94.5$, $P = 0.89$), nor in the total number of flowers probed per bout during the observations (L-morph = 93.7 ± 71.5 , $N = 15$ plants; S-morph = 102.5 ± 105.5 , $N = 13$ plants; $U = 97.0$, $P = 0.98$). The number of flowers probed per foraging bout ranged from 1 to 65, and in most cases (71%), an individual forager probed <20 flowers per bout. The smallest but least frequent hummingbird (*Aethopyga heloisa*) probed >20 flowers per bout, followed by the most frequent floral visitors, *A. cyanocephala* and *C. thalassinus* (Table 2). However, floral morphs did not differ in the number of flowers probed per foraging bout (L-morph = 16.2 ± 13.1 , $N = 83$ plant visits; S-morph = 16.8 ± 14.3 , $N = 79$ plant visits; $U = 94.0$, $P = 0.87$) or in the number of flowers visited per hour (L-morph = 2.9 ± 1.1 , $N = 15$ plants; S-morph = 3.2 ± 2.0 , $N = 13$ plants; $U = 94.0$, $P = 0.87$). Foraging activity peaked between 0930 and 1030 (Fig. 5).

We observed the 11 hummingbird species known to visit *P. padifolia* (Table 2). Solitary bees, *Plebeia mexica* Ayala and *Thygater* sp.), bumble bees (*Bombus medius* Cresson [Apidae]), and butterflies (*Heliconius hortense* and *H. ismenius*

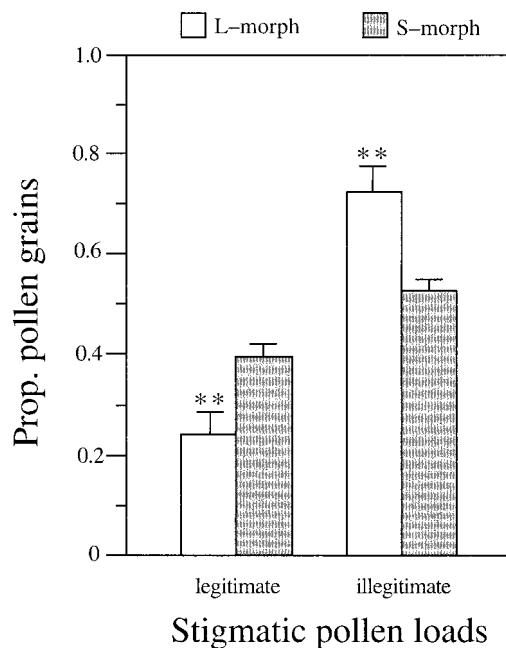


Fig. 3. Proportion of legitimate and illegitimate pollen classified by size on stigmas of L-morph and S-morph of *Palicourea padifolia* flowers. Data are means + 1 SE. One-way ANOVAs, legitimate, $F_{1,48} = 8.6, P = 0.005$; illegitimate, $F_{1,48} = 12.1, P = 0.001$.

[Nymphalidae: Heliconiinae]) also visited during our focal observations, but their frequency was much lower than hummingbirds' visitation (Fig. 5). *Heliconius* butterflies mainly fed on pollen. Pollen-collecting bees were observed visiting flowers legitimately, and nectar robbing by *Polistes* wasps and *Trigona* bees was also common. The most common hummingbird species was *Amazilia cyanocephala* (30.6% of total visits), followed by *A. beryllina* and *Colibri thalassinus* (16.6 and 11.7%, respectively). Butterflies represented 10% of the visits, and 5% were by bumble bees and 2.8% by unidentified bees (Table 2). Other uncommon hummingbird species accounted for the remaining visits (23.3%).

Movements from focal plants toward neighboring plants were only recorded for hummingbirds ($N = 170$). Of the observed individuals, 14% (13% for L-morph plants vs. 15% S-morph plants) flew to another plant of the opposite floral morph, 16% (19 vs. 12) to another plant of the same floral morph, 18% (21 vs. 14) towards a branch where it perched for several minutes until foraging again, and 52% (47 vs. 59) flew out of sight. Frequencies of these behavioral outcomes were independent of floral morph ($\chi^2 = 3.12, df = 3, P > 0.05$).

Pollen donation capabilities of hummingbirds—Variation in pollen number differed among morph combinations in the pollen transfer experiment with stuffed hummingbirds (two-way ANOVA; morph combination effect, $F_{3,60} = 4.15, P = 0.0097$). Pollen from S-morph flowers was deposited significantly more on stigmas of L-morph flowers than on S-morph stigmas. In contrast, pollen from L-morph flowers was deposited equally on stigmas of both morphs (Fig. 6). Although pollinator effect was marginally significant on pollen receipt (pollinator effect; $F_{2,60} = 2.69, P = 0.075$), hummingbirds deposited significantly fewer pollen grains between S-morph flowers than they did between other morph combinations (post

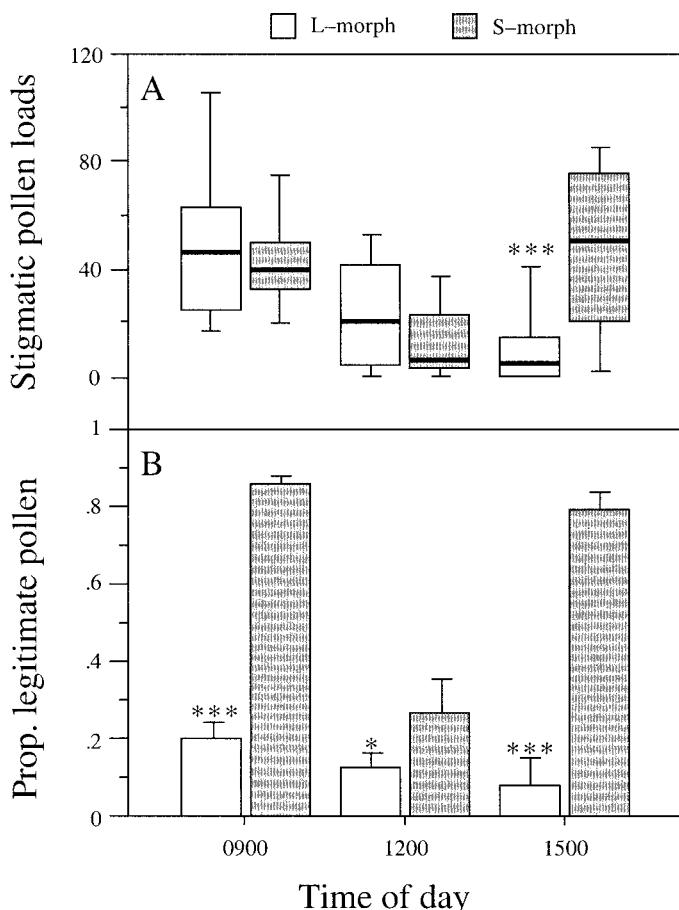


Fig. 4. Rates of pollen deposition on stigmas by (A) number of pollen grains per stigma and (B) proportion of legitimate pollen of L- and S-morph flowers throughout the day in *Palicourea padifolia*. Box plots show the distribution of values for individual flowers within each morph: thick bar indicates median (50th percentile), box indicates 25th and 75th percentiles, and thin bar indicates 10th and 90th percentiles. * $P < 0.05$, *** $P < 0.001$.

hoc mean comparisons, $P < 0.05$). The morph combination \times pollinator interaction was not statistically significant ($F_{6,60} = 0.25, P = 0.95$). On average, long-billed hummingbird, *Eugenes fulgens*, transferred more pollen grains across all morph combinations than did *Amazilia cyanocephala* (medium) and *Atthis heloisa* (small), but differences in mean stigmatic pollen loads were only statistically significant between *E. fulgens* and *A. cyanocephala* (post hoc mean comparisons, $F_{1,1} = 5.35, P = 0.0241$; Fig. 7).

Pollination effectiveness of hummingbirds—A total of 180 flowers was pollinated with stuffed hummingbirds, 30 exposed to natural pollination, and 30 not pollinated and excluded from pollinators (240 experimental flowers). We lost 70 of them over the experiment. Of those that survived (170), only 40% of those pollinated with stuffed hummingbirds and 50% naturally exposed to pollinators had developed fruits 2 mo after pollination. None of the control flowers excluded from pollinators developed fruits.

A G test of independence with Williams' correction indicated independence of fruit production from pollinator species and pollen flow direction (intermorph) in flowers with one ($G = 0.4522, df = 2, P > 0.05$) or five probes ($G = 1.636, df$

TABLE 2. Floral visitors of *Palicourea padifolia* (Rubiaceae).

Species	Body mass (g)	Length of exposed culmen (mm)	No. visits (%)	No. flowers probed/foraging bout (mean \pm SD)
Hummingbirds				
<i>Campylopterus curvipennis</i>	9.2	27.2	6 (3.3)	6.3 \pm 5.3
<i>C. hemileucurus</i>	11.0	27.8	7 (3.9)	10.7 \pm 4.8
<i>Colibri thalassinus</i>	5.0	20.9	21 (11.7)	21.1 \pm 14.9
<i>Anthracothorax prevostii</i>	6.9	27.1	1 (0.5)	10
<i>Amazilia candida</i>	3.6	17.2	4 (2.2)	23.0 \pm 28.7
<i>A. cyanocephala</i>	4.7	19.7	55 (30.6)	17.8 \pm 13.3
<i>A. beryllina</i>	4.2	19.5	30 (16.7)	18.5 \pm 12.3
<i>A. yucatanensis</i>	4.0	20.8	2 (1.1)	14.0 \pm 1.4
<i>Lamprolais amethystinus</i>	5.2	22.5	7 (3.9)	5.3 \pm 5.2
<i>Eugenes fulgens</i>	6.8	29.5	7 (3.9)	13.6 \pm 12.7
<i>Atthis heloisa</i>	2.2	12.4	8 (4.4)	24.5 \pm 20.4
Insects				
<i>Heliconius hortense</i>			18 (10)	4.8 \pm 2.9
<i>Plebeia mexica</i>			5 (2.8)	5.0 \pm 4.6
<i>Bombus medius</i>			9 (5)	11.0 \pm 9.1

= 2, $P > 0.05$). Because the three-way interaction (pollination treatment \times pollen flow direction \times fruit production) was not significant, we pooled our data from the two pollination treatments and carried out two-way G tests of independence. Fruit production was not independent of pollen flow direction ($G = 3.80$, $P = 0.05$); S-morph flowers developed almost twice the number of fruits developed by L-morph flowers (Table 3), and the proportion of fruits developed by S-morph flowers was significantly higher than the proportion developed by L-morph

flowers ($Z = 2.03$, $P < 0.05$). Those differences cannot be accounted for by corolla size variation of either the receptive (two-way ANOVA, floral morph \times fruit production interaction, $F_{1,178} = 1.08$, $P = 0.29$) or the donor flower (floral morph \times fruit production interaction, $F_{1,134} = 1.38$, $P = 0.24$).

DISCUSSION

Hummingbird effectiveness as pollen vectors—Pollinators have been considered as the main evolutionary force in the process of gender specialization in distylous species (Ornduff, 1975; Charlesworth and Charlesworth, 1979; Lloyd, 1979; Barrett, 1992, and references therein). Although there is some experimental evidence suggesting that hummingbirds of dif-

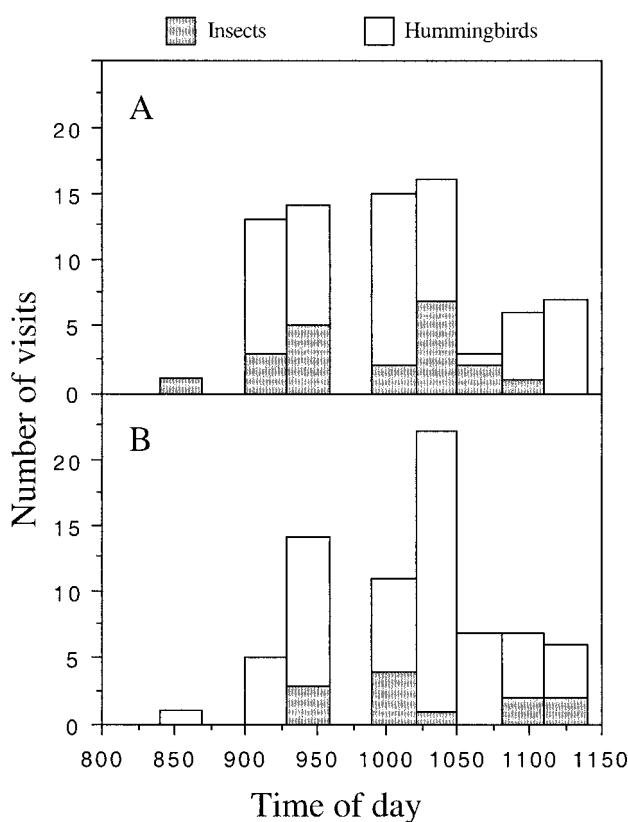


Fig. 5. Visitation frequency by insects and hummingbirds in (A) long-styled and (B) short-styled plants of *Palicourea padifolia*.

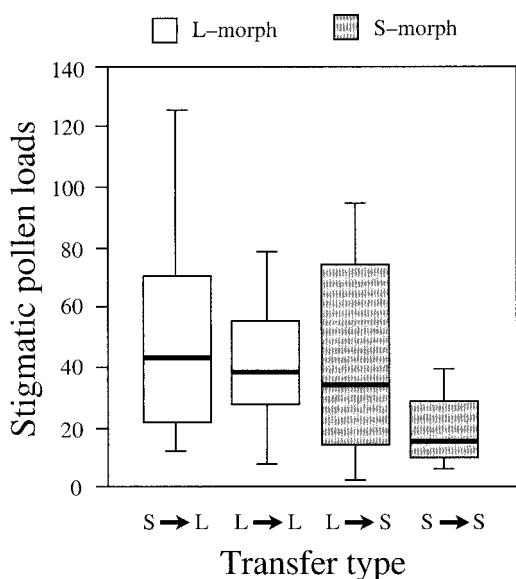


Fig. 6. Stigmatic pollen loads showing all combinations of inter- and intramorph pollen transfer efficiency of stuffed hummingbirds. Box plots show the distribution of values for individual transfers within each morph combination: thick bar indicates median (50th percentile), box indicates 25th and 75th percentiles, and thin bar indicates 10th and 90th percentiles. S = short-styled, L = long-styled.

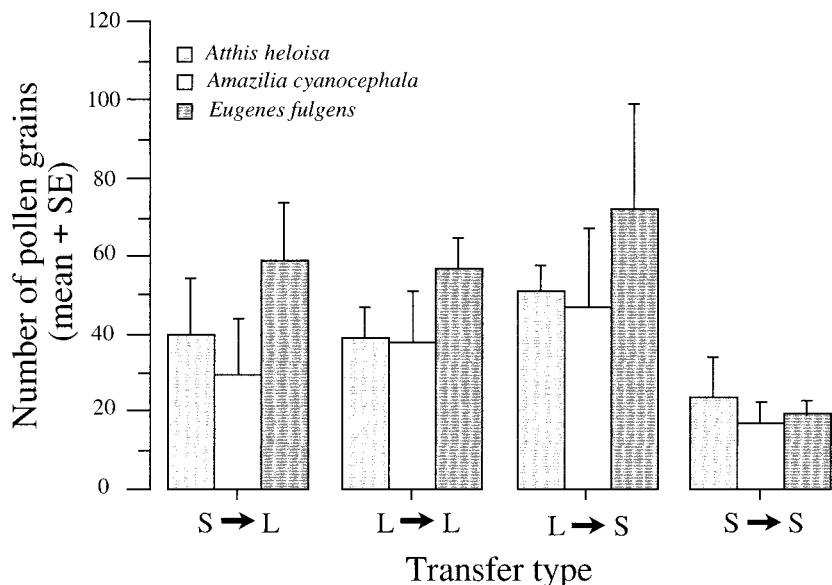


Fig. 7. Mean number of pollen grains of *Palicourea padifolia* deposited on stigmas of L-morph and S-morph flowers by stuffed hummingbirds. S = short-styled, L = long-styled.

ferent species place pollen differently (Campbell et al., 1994; Temeles and Rankin, 2000), there are few empirical studies of distylos, hummingbird-pollinated species. Pollinator effectiveness (pollen transfer between opposite floral morphs) and variation in pollen placement on pollinators' bodies to explain the observed differences in reproductive output have been infrequently explored (e.g., Feinsinger and Busby, 1987; Feinsinger et al., 1988; Stone and Thomson, 1994; Stone, 1995, 1996; Lau and Bosque, 2003), and in particular, how differences in morphology and behavior on distylos species by a suite of pollinator species could explain the variation in the expression of heterostyly (Faivre and McDade, 2001). We acknowledge the limitation of our study in terms of assuming that hummingbirds are the most important pollen vectors. Pol-

len removal by pollen-collecting insects (<15% of visits) from the S-morph anthers of *P. padifolia* needs further study as insects effectively remove pollen grains in other distylos species (Wolfe and Barrett, 1987; Wesselingh et al., 2000).

Hummingbirds often obtain nectar from distylos flowers, but their effectiveness in dispersal and transfer of legitimate pollen (intermorph) has been rarely investigated for distylos plants (Feinsinger and Busby, 1987; Feinsinger et al., 1988; Stone and Thomson, 1994; Stone, 1995; Murcia and Feinsinger, 1996; Lau and Bosque, 2003). The effectiveness of legitimate, symmetrical pollen transfer by hummingbirds depends on their matching of their head morphology to the architecture of a plant's sexual structures (Murcia and Feinsinger, 1996). Our data suggest that the differences in pollen transfer and fruit production cannot solely be accounted for by the pollinators' morphological matching. In fact, intermorph pollen transfer and fruit production favored S-morph plants, independently of hummingbird species. The most frequent, medium-billed floral visitor, *A. cyanocephala*, was the least effective pollinator in *P. padifolia*. However, pollinator behavior may limit seed set in plants when the number of pollinator movements between flowers on the same plant is larger compared to the number of movements between individuals. That results in higher levels of geitonogamous pollination, lowering the probability of successful fertilization by clogging the stigma and style with incompatible pollen tubes (reviewed by Lloyd and Webb, 1992; Ree, 1997). Thus, seed set would be depressed even if stigmas received an excess of compatible pollen grains relative to ovules. We observed no differences in pollinator movements between morphs. Nor did we detect differences in the number of visits to a given plant nor in the number of flowers visited. Nevertheless, individual foragers probed approximately 20 flowers per foraging bout, and that surely resulted in geitonogamous crosses. Although geitonogamous pollination was out of the scope of this study, further research is needed to determine whether the clogging of stigma and style with incompatible pollen tubes lowers the probability of successful fertilization between floral morphs.

TABLE 3. Fruit set results from intermorph pollinations of *Palicourea padifolia* with stuffed hummingbirds. N = number of pollinated flowers. Fruit set was calculated from the total number of flowers experimentally pollinated. S = short-styled, L = long-styled.

Cross	N	No. fruits developed	Fruit set (%)
<i>Atthis heloisa</i>			
S → L	20	4	20.0
L → S	22	9	40.9
<i>Amazilia cyanocephala</i>			
S → L	16	5	31.2
L → S	26	14	53.8
<i>Eugenes fulgens</i>			
S → L	19	8	42.1
L → S	23	11	47.8
Totals			
S → L	55	17	30.9
L → S	71	34	47.9
Control unbagged			
L	9	2	22.2
S	13	4	30.8

Other aspects of hummingbird foraging behavior may be important to explain asymmetry in pollen loads leading to differences between floral morphs in male reproductive success and the overall reproductive output. Pollen grains can be “wasted” by active removal and territoriality of hummingbirds. Territorial hummingbirds (*Amazilia cyanocephala* and *A. yucatanensis*) have been commonly observed cleaning their bills in *P. padifolia* between foraging bouts by using their feet or by rubbing their bills against a branch (Ree, 1997; Contreras and Ornelas, 1999; see also Feinsinger et al., 1988; Wolfe and Barrett, 1989). A more quantitative approach is needed to elucidate the effect of territorial foraging on pollen flow and seed set in *P. padifolia*.

Reversed stigma dimorphism—Floral morphs may exhibit gender specialization and gain differential reproductive success through male and female function, and heterostyly could evolve into dioecy when pollen transfer between floral morphs is highly asymmetrical (Casper, 1992 and references therein). Pollinators have been emphasized as the main causal agents of the evolutionary breakdown of heterostyly. However, other selective forces may be operating on the reproductive system of *P. padifolia*, and gender specialization as a result of genetic and developmental pathways may lead to male function sterilization (Domínguez et al., 1997). Baker (1958) and Ornduff (1966) suggested that heterostylous plants might become functionally dioecious if pollen is more effectively transferred in one direction. Three, non-mutually exclusive explanations for the observed differences between floral morphs in fruit output from our controlled pollination experiment are possible: (1) male sterility among short-styled individuals, (2) more effective transfer of legitimate pollen by pollinators from short-styled to long-styled flowers, and (3) more effective adherence of pollen on the larger receptive surface of stigmas in short-styled flowers to pollinators.

In some distylous species, pollen loads on S-morph stigmas have been observed to contain a greater proportion of intermorph pollen than pollen loads on L-morph stigmas (Feinsinger and Busby, 1987; Lau and Bosque, 2003), as observed also in *P. padifolia*. Indeed, our data have shown that S-morph stigmas ended with more legitimate pollen grains than L-morph stigmas. This has been attributed to differences between floral morphs in their effectiveness in depositing and removing pollen from the bodies of pollinators and to the larger numbers of pollen grains produced by L-morph anthers compared with S-morph (Ganders, 1979; Piper and Charlesworth, 1986). Some authors have suggested that intermorph differences in stigma sizes may be important in determining the nature of stigmatic pollen loads (Schou, 1983; Dulberger, 1992; Ree, 1997; Lau and Bosque, 2003) and a determinant of relative reproductive fitness of a particular morph (Dulberger, 1992).

Stigmas of L-morph and S-morph flowers of distylous species commonly differ in their size and the size and/or shape of their papillae (Vuilleumier, 1967; Ganders, 1979; Dulberger, 1992; Dulberger and Ornduff, 2000; Faivre and McDade, 2001). Also, marked dimorphisms in stigma shape are known in distylous species (Hermann et al., 1999; Dulberger and Ornduff, 2000 and references therein). The stigma-size dimorphism has been functionally interpreted as a way to increase receptive area enhancing intermorph pollen flow in the less accessible S-morph stigmas (Dulberger, 1992). In contrast, the significance of the stigma-shape dimorphism in the distylous polymorphism is obscure (Hermann et al., 1999). The stig-

matic-length difference between floral morphs is high in *P. padifolia* compared to those recorded for distylous species (Dulberger, 1992; Dulberger and Ornduff, 2000). The ratio of L-morph to S-morph stigma length was 3.4. It is noteworthy that in all other species studied, L-morph stigmas are larger than S-morph stigmas (Ganders, 1979; Dulberger and Ornduff, 2000), a condition opposite to that of many distylous *Palicourea* species (Sobrevila et al., 1983; Feinsinger and Busby, 1987; Taylor, 1993, 1997; Ree, 1997). For known distylous *Palicourea* species, stigmas of L-morph plants are usually 0.5–1 mm long, while those of S-morph plants are generally 1–3 mm long and always larger than those of L-morph plants (C. M. Taylor, Missouri Botanical Garden, personal communication). This apparent “reversed” stigma dimorphism is often accompanied with asymmetry in pollen transfer in distylous *Palicourea* species that have been studied in detail, in which S-morph individuals received more pollen that is legitimate (Feinsinger and Busby, 1987; Lau and Bosque, 2003; this study), and greater female reproductive success than L-morph individuals (this study). Dulberger and Ornduff (2000) suggested that the different configurations of L- and S-morph stigmas maximize their abilities to remove intermorph pollen distributed by L- and S-morph anthers in differing patterns on a pollinators’ body (see also Hermann et al., 1999). We cannot evaluate the functional significance of the apparent differences in stigma shape and stigmatic papillae between floral morphs, because papillae number and stigma shape were not quantified. Also, various bill morphologies were equally effective in depositing and removing pollen in both directions. However, our study suggests that the differences in stigmatic pollen loads, both under field and controlled conditions, cannot solely be the result of pollen and stigma dimorphisms because of different rates of pollen accumulation between floral morphs throughout the day. It is possible that a nonevaluated behavioral component of hummingbird foraging may explain in part the different rates of pollen accumulation in *P. padifolia* over time. We have shown (Ornelas et al., 2004) that S-morph flowers contained significantly more nectar (nectar standing crops) early in the morning than L-morph flowers. These data suggest that hummingbirds are visiting earlier L-morph flowers with significantly higher nectar volumes than S-morph flowers. Nevertheless, S-morph plants developed more fruits than L-morph plants after 2 mo of pollination with stuffed hummingbirds. Stigma and pollen dimorphisms accompanied with pollinator behavior may play a functional role in facilitating legitimate pollination in one direction and explain in part the higher relative reproductive success of S-morph plants, as suggested by our pollination experiment and patterns of fruit production of natural-pollinated plants (Ornelas et al., 2004).

The Neotropical genus *Palicourea* Aublet (Rubiaceae) (approximately 200 species) is composed of mostly hummingbird-pollinated distylous species, which appears to be the ancestral condition for the genus, and lost in some species on Caribbean islands (Taylor, 1993, 1997). A comparative phylogenetic study is needed to establish whether the stigma-size dimorphism is a morph reversal among distylous *Palicourea* species and how floral traits that define the distylous polymorphism evolved in the context of hummingbird pollination.

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