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Long-and short-billed hummingbirds as pollinators of *Palicourea demissa*, a distylous treelet of Neotropical cloud forests

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Abstract

Distyly has been interpreted as a mechanism that promotes cross-pollination between floral morphs. According to this hypothesis, pollen from anthers positioned at different heights could adhere to different body parts of the pollinator that would correspond to those points where stigmas of compatible morph contact the animal. In this regard, hummingbird species with different bill sizes may play different roles as pollinators of each morph. If pollinators mobilize more legitimate pollen towards one of the two morphs, gender specialization may occur. This work aimed to assess experimentally the role of long- and short-billed hummingbirds as pollinators of short-style (SS) and long-style (LS) flowers of *Palicourea demissa*, a distylous, hummingbird-pollinated treelet in Venezuelan cloud forests. Flowers were emasculated and exposed to a single visit of the hummingbird *Coeligena torquata* (long-billed), *Heliangelus spencei* (short-billed) or *Adelomyia melanogenys* (short-billed). Later, stigmas were removed, and pollen load counted under a microscope to calculate the probability of legitimate- and illegitimate-pollen transfer by hummingbirds. The probability analyses of pollen transference showed that short-billed hummingbirds have higher pollination probabilities from SS-anthers to LS- and SS-stigmas, and from LS-anther to LS-stigmas than from LS-anther to SS-stigmas. In contrast, long-billed hummingbirds have higher probabilities of pollen transference from LS-anthers to SS-stigmas than in other directions. A deeper view of the sexual expression of each morph in *P. demissa* will depend on future studies that determine possible morpho-differences in the biological function of male and female floral structures, and the role played by less frequent floral visitors as mediators of legitimate pollination between floral morphs.

Keywords Cross-pollination · Floral morphs · Gender specialization · Heterostyly · Pollen flow

Introduction

Distyly is a genetically controlled polymorphism where two floral morphs coexist in the same population (Barrett 2002; Darwin 1877). The distylous populations are characterized by having plants that display flowers with long styles and short stamens (hereafter LS) and plants that exhibit flowers with the opposite pattern (hereafter Barrett 2002). Besides the reciprocal position of anthers and stigmas (reciprocal herkogamy), floral morphs of distylous plants exhibit other polymorphisms in anthers, stigmas, and pollen grains, such as anther and stigmatic papillae size and shape, pollen grain size and sculpture, and generally are self- and intramorph incompatible (Barrett 1990, 1992, 2002; Barrett and Richards 1990; Barrett et al. 2000; Richards and Barrett 1992).

Darwin (1877) suggested reciprocal herkogamy as a mechanism that promotes cross-pollination among conspecific plants by pollinators. According to Darwin´s hypothesis, pollen from anthers positioned at different heights could adhere to different body parts of the pollinator that would correspond to those points where stigmas of the compatible morph contact the animal (Barrett 2002; De Jong and Klinkhamer 2005). Although reciprocal herkogamy promotes cross-pollination, some studies show that floral morphs may differ in their male (pollen donation) and female (pollen reception and fruits and seeds production) reproductive functions (García-Robledo 2008; González et

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al. 2005; Ornelas et al. 2004a; Valois-Cuesta et al. 2011a, b, 2012), suggesting that the effectiveness of legitimate pollen transfer between floral morphs (from SS to LS and from LS to SS flowers) depends of the foraging behaviour of pollinators, and how well aligned are flowers and pollinators morphologies (Beach and Bawa 1980; Furtado et al. 2021; Keller et al. 2014; Morales et al. 2012; Ornelas et al. 2004a; Raupp et al. 2020).

Hummingbirds are important pollen vectors and pollinators in most distylous species with tubular-type flowers (Bergamo et al. 2019; Hernandez and Ornelas 2007; Machado et al. 2010; Maruyama et al. 2016). However, hummingbird species may play different roles as pollinators if they vary in bill size (Furtado et al. 2021; Lau and Bosque 2003; Morales et al. 2012; Ornelas et al. 2004b). If pollinators transport more legitimate pollen towards one of the two morphs, gender specialization may occur (Beach and Bawa 1980). Long- and short-billed hummingbirds may disrupt balanced intermorph pollen flow resulting in unidirectional pollen flow; thus, floral morphs may gradually specialize as either male or female (Beach and Bawa 1980). Functional dioecy or evolution of dioecy from distyly has been recorded in some species, especially of the Rubiaceae family (Duan et al. 2018; Liu et al. 2012; Ornelas et al. 2020; Watanabe et al. 2014).

Palicourea demissa Standl. (Rubiaceae) is a distylous, hummingbird-pollinated treelet of Neotropical cloud forests. In *P. demissa*, long-billed and short-billed hummingbirds pollinate plants of both morphs. However, SS-morph receives more legitimate pollen and produces more fruit and viable seeds than LS-morph (Valois-Cuesta et al. 2011b), suggesting that hummingbirds perhaps promote gender specialization in each floral morph (Valois-Cuesta et al. 2012). On this basis, we formulate the following research questions: Can long- and short-billed hummingbirds promote asymmetric pollen flow between floral morphs of *P. demissa*? If hummingbirds disrupt the complementarity of legitimate pollen transfer between floral morphs would gender specialization in *P. demissa* be expected? We hypothesized that differences in bill size of hummingbirds may promote asymmetric pollen flow between morphs of *P. demissa* and it may lead to gender specialization. This research aimed to assess experimentally the role of long- and short-billed hummingbirds as mediators of legitimate pollination between floral morphs of *P. demissa* in a Venezuelan cloud forest.

## Materials and methods

Study area

Fieldwork was carried out in La Mucuy Bird Observatory (8º 38´ N, 71º 02´ W; at 2,300-2,400 m a.s.l.), Sierra Nevada National Park, Mérida, Venezuela. In La Mucuy cloud forest, the mean annual temperature is 14 ºC, and mean annual precipitation varies between 2,800 and 3,400 mm with peaks in April-May and October-November. Horizontal precipitation (fog) represents about 300 mm (Ataroff and Rada 2000). The La Mucuy cloud forest is rich in vascular epiphytes (Ataroff and Sarmiento 2004) and bird species; about 14 hummingbird species have been recorded (Rengifo et al. 2005).

Study species

Palicourea demissa is a distylous, hummingbird-pollinated treelet (3–8 m in height) commonly found in the Andean cloud forests of Colombia, Venezuela and Perú. The peak of flowering occurs between May and June; during this time, each plant displays 23.4 ± 5.92 inflorescences and each inflorescence opens 2.4 ± 0.1 flowers per day (Valois-Cuesta et al. 2011a). The life span of individual inflorescences from the first open flower until fruit initiation of the last open flower is about 47.6 ± 3.0 days (Valois-Cuesta et al. 2011b). Flowers are pedicellate, gamopetalous and arranged on an elongate central axis (rachis) of reddish color (Fig. 1). Anthesis starts between 05:30 and 07:30 h and lasts c. 14 h (Valois-Cuesta and Novoa-Sheppard 2006; Valois-Cuesta et al. 2011b). The SS-flowers display larger corollas, stigmatic lobes, anthers, pollen grains, and anthers-stigma separation (herkogamy) than LS-flowers, but the reciprocity among sexual organs is higher between LS-anthers and SS-stigmas than between SS-anthers and LS-stigmas (Valois-Cuesta et al. 2011a).

Flowers are mainly visited by six hummingbird species (94.8% of visits): *Coeligena torquata* (34.3%), *Heliangelus spencei* (22%), *Adelomyia melanogenys* (19.1%), *Aglaiocercus kingi* (7.2%), *Ocreatus underwoodii* (6.5%) and *Colibri thalassinus* (5.6%). Insects represent 5.3% of visits (Valois-Cuesta et al. 2011b). Floral morphs display a 1:1 morph ratio (Valois-Cuesta and Novoa-Sheppard 2006) and do not differ in the number of pollen grains and ovules per flower or the number of flowers per inflorescence. Plants are self- and intramorph incompatible (Valois-Cuesta et al. 2011a). Fruit set did not differ between artificially outcross-pollinated LS and SS flowers. However, under natural conditions, SS-plants produce 20% more fruits than LS-plants (Valois-Cuesta et al. 2011a). Thus, a discrepancy between

Fig. 1 *Palicourea demissa* (Rubiaceae): SS- and LS-inflorescences with opening flowers and floral bud **(a, b)**, SS-and LS flowers **(c, d)**, and SS-and LS stigmas **(e, f)**. Scale bars: 1 cm in **a**, **b** and **d**; 1 mm in **c**, **e** and **f**
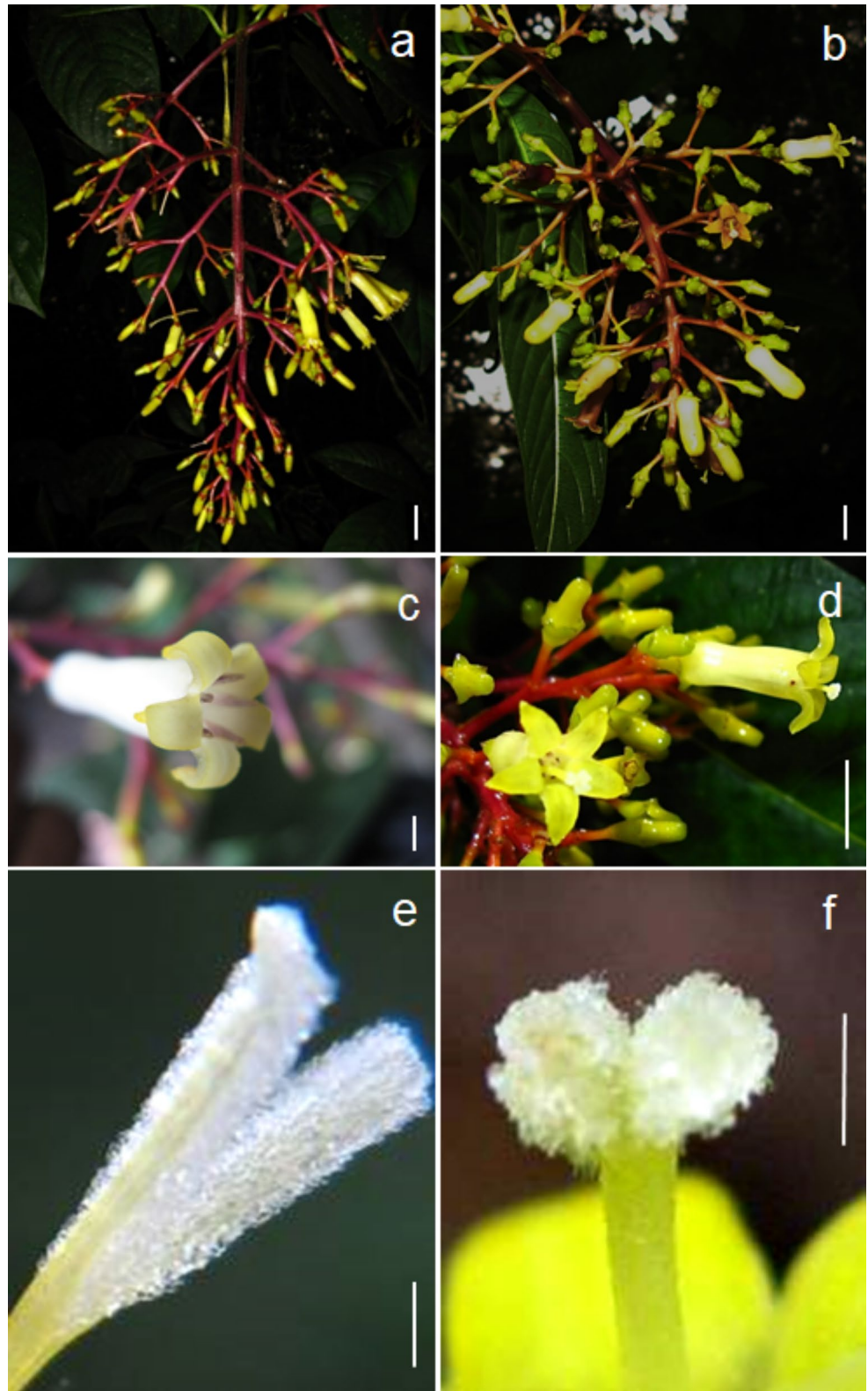
the potential and realized functional gender does occur (Valois-Cuesta et al. 2012).

Pollen deposition by long-and short-billed hummingbirds

We selected 90 flowers in pre-anthesis from 30 plants

between March and June 2008: three flowers from each of 15 plants for each morph. Flowers in pre-anthesis were excluded with tulle bags to avoid floral visits. Later, under natural conditions, flowers were emasculated by removing the anthers using fine forceps and exposed to a single visit of a hummingbird species (15 flowers per morph per hummingbird species), during the time of day when the highest visitation rate by hummingbirds occurs (between 09:00 and 12:00; Valois-Cuesta and Novoa-Sheppard 2006; Valois-Cuesta et al. 2011b). Three hummingbird species were considered to pollinate flowers of both morphs: *C. torquata* (long-billed, 33 mm), *H. spencei* (short-billed, 18 mm) and *A. melanogenys* (short-billed, 13 mm; Figs. 2 and 3). Pollinated stigmas were carefully removed of the flowers, immediately fixed on a microscope slide with basic fushin gel and observed under a Leitz Dialux 20 EB microscope (Kearns and Inouye 1993). We proceeded to count the pollen grains of each morph with a manual counter. The magnitude of legitimate pollination in both morphs was assessed by counting the number of pollen grains of the opposite morph on the stigma in both morphs. We assumed that pollen grains with a diameter > 105 μm arrived from SS-flowers and those < 100 μm arrived from LS-flowers (Fig. 2).

To compare the capacity of each hummingbird species as a mediator of legitimate and illegitimate pollination we followed Lloyd and Webb (1992):

$$Tij = \frac{(\text{Average stigmatic pollen load) ij} \times (\text{Number of flowers) j}}{(\text{Number of pollen per flower) i} \times (\text{Number of flowers) i}} \quad (1)$$

where Tij represents the probability of transfer of pollen type i to a stigma j and i and j are the donor and recipient morphs, respectively. Data on the number of pollen grains per flower were obtained from Valois-Cuesta et al. (2011a).

In addition, we calculated the relative importance of pollen flow (RIPF: LS→SS, LS→LS, SS→SL or SS→SS) promoted by each hummingbird species with the following equation:

$$RIPF = \frac{Tij_a}{Tij_a + Tij_b + Tij_c + Tij_d} \times 100 \quad (2)$$

where RIPF represents the relative importance of the transfer probability of a particular pollen flow (Tij_a) compared to the sum of all transfer probabilities ($Tij_a + Tij_b + Tij_c + Tij_d$). For example, the relative importance of pollen flow (RIPF) LS → SS is equal to the transfer probability LS → SS divided by the sum of all the transfer probabilities of LS→SS + LS→LS + SS→SL + SS→SS. The values of relative importance are presented in percentages of pollen transfer.

Statistical analyses

To evaluate the floral morph effect on pollen reception (total, alien "another species", *P. demissa* "legitimate and illegitimate") and the hummingbird-species (or bill size) effect on their capacities of pollen deposition on virgin stigmas of both floral morphs, generalized linear mixed-effects models (GLMM) were used. In the models, floral morph and hummingbird species were considered the main factors and the different pollen load metrics were included as response variables. Along with the main effects, a floral morph × hummingbird species (or bill size) interaction was included in the model. Differences between morphs in pollen loads on virgin stigmas deposited by hummingbird species were evaluated using Mann-Whitney test. To carry out a parametric statistical test (GLMM) data were square root-transformed (x + 0.5) to correct normality and homoscedasticity. However, untransformed data (mean ± 2 SE) are reported in text, tables and figures. All analyzes were performed in the computer program R (R Core Team 2021) using the package "nlme" Version 3.1–137 (Pinheiro et al. 2018).

Results

The total pollen load deposited on stigmas after a single visit of a pollinator hummingbird was not significantly different between floral morphs (SS = 13.2 ± 8.3, LS = 13.7 ± 8.4; $F_{1,84} = 0.02$, $P = 0.89$) nor among hummingbird species (*C. torquata* = 8.0 ± 4.4, *H. spencei* = 19.4 ± 12.1, *A. melanogenys* = 12.8 ± 12.0, $F_{2,84} = 2.05$, $P = 0.13$). However, the pollen load deposited on stigmas of each morph was not independent of the hummingbird species (floral morph × hummingbird species interaction, $F_{2,84} = 4.15$, $P = 0.02$, Table 1). Similarly, the number of pollen grains of *P. demissa* on stigmas did not vary significantly between floral morphs ($F_{1,84} = 0.70$, $P = 0.40$) or among hummingbird species ($F_{2,84} = 2.11$, $P = 0.12$), but the floral morph × hummingbird species interaction was statistically significant ($F_{2,84} = 5.78$, $P = 0.004$). According to this, *H. spencei* deposited more pollen grains of *P. demissa* on LS-stigma than on SS-stigma, while *C. torquata* and *A. melanogenys* deposited more conspecific pollen on SS-stigma than on LS-stigma (Table 1). The floral morph ($F_{1,84} = 0.04$, $P = 0.84$) and hummingbird species effect ($F_{2,84} = 1.69$, $P = 0.19$), and its interaction (floral morph × hummingbird species) were not significant for the number of alien pollen ($F_{2,84} = 2.13$, $P = 0.12$; Table 1; Fig. 4).

The number of illegitimate pollen grains deposited on stigmas was similar between floral morphs (SS = 3.5 ± 1.6, LS = 4.2 ± 3.9, $F_{1,84} = 0.27$, $P = 0.60$), but significantly different among hummingbird species (*C. torquata* = 1.4 ± 1.0,
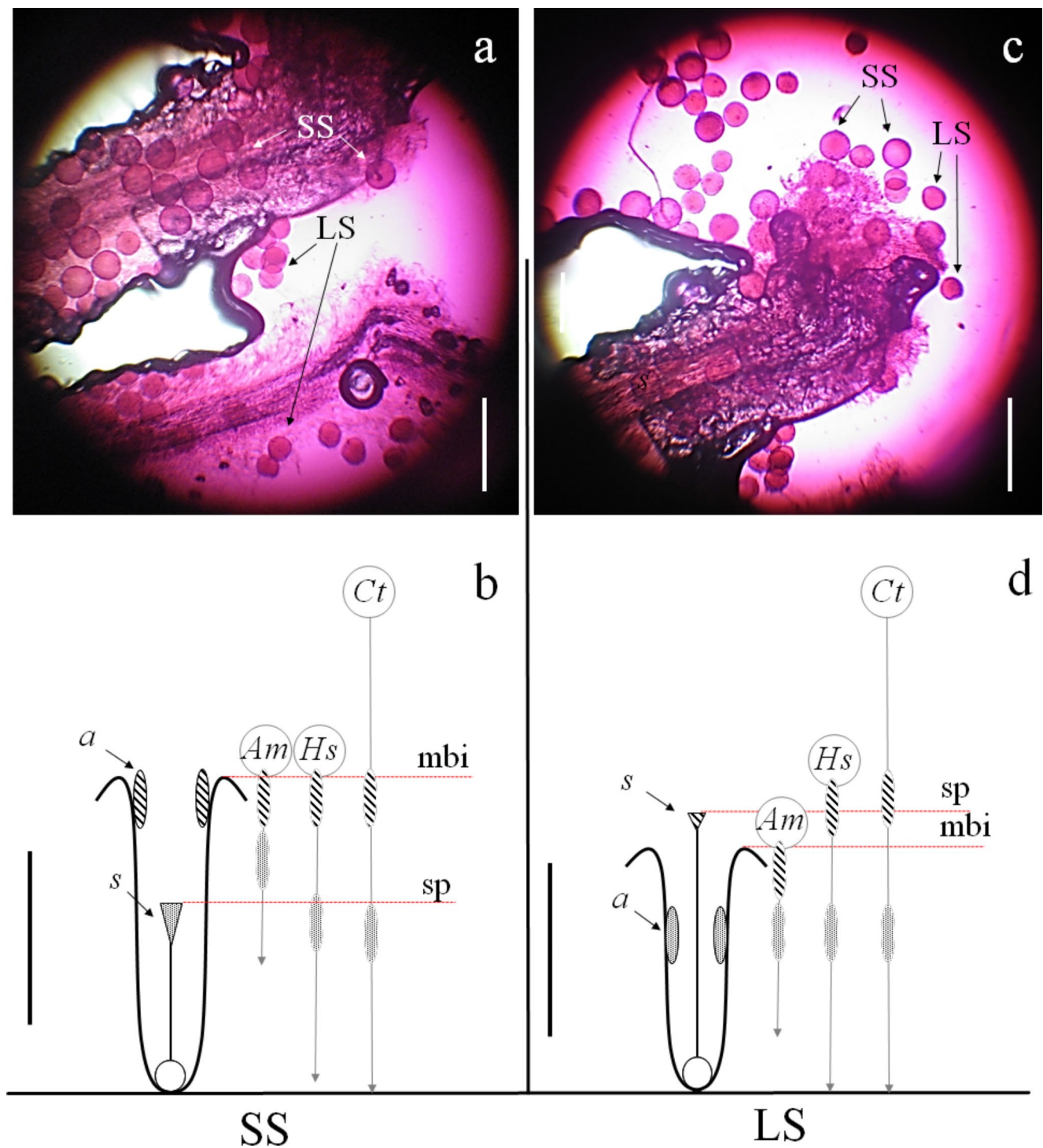
Fig. 2 SS-and LS pollen grains on stigmas of both morphs of *Palicourea demissa* (**a**, **c**). Schematic figure showing the relative size and position of corolla, stigmas (sp), anthers (**a**), and maximum bill insertion of hummingbird species (mbi) *Adelomyia melanogenys* (*Am*), *Heliangelus spencei* (*Hs*) and *Coeligena torquata* (*Ct*) into the corolla of floral morphs (**b**, **d**). Bar = 500 μm in **a** and **c**; 1 cm in **b** and **d**

H. spencei = 7.9 ± 5.7, *A. melanogenys* = 2.2 ± 1.8, $F_{2,84}$ = 7.01, $P = 0.001$). The floral morph × hummingbird species interaction was not significant ($F_{2,89} = 2.12$, $P = 0.12$; Table 1). On the other hand, the number of legitimate pollen grains deposited on stigmas was not different between floral morphs (SS = 4.7 ± 2.6, LS = 8.1 ± 4.8; $F_{1,84} = 1.56$, $P = 0.21$) or among hummingbird species (*C. torquata* = 5.7 ± 4.1, *H. spencei* = 10.1 ± 6.7, *A. melanogenys* = 3.4 ± 1.9; $F_{2,84}$ = 2.00, $P = 0.14$). However, the floral morph × hummingbird

Fig. 3 Most frequent flower visitors and pollinators of SS- and LS flowers in *Palicourea demissa* (Rubiaceae): *Adelomyia melanogenys***(a, b)**, *Heliangelus spencei***(c, d)** and *Coeligena torquata***(e, f)**. In italic: anthers **(a)**, stigma (*s*), and pollen adhered to the hummingbird's bill (*pg*). Scale bars 1 cm
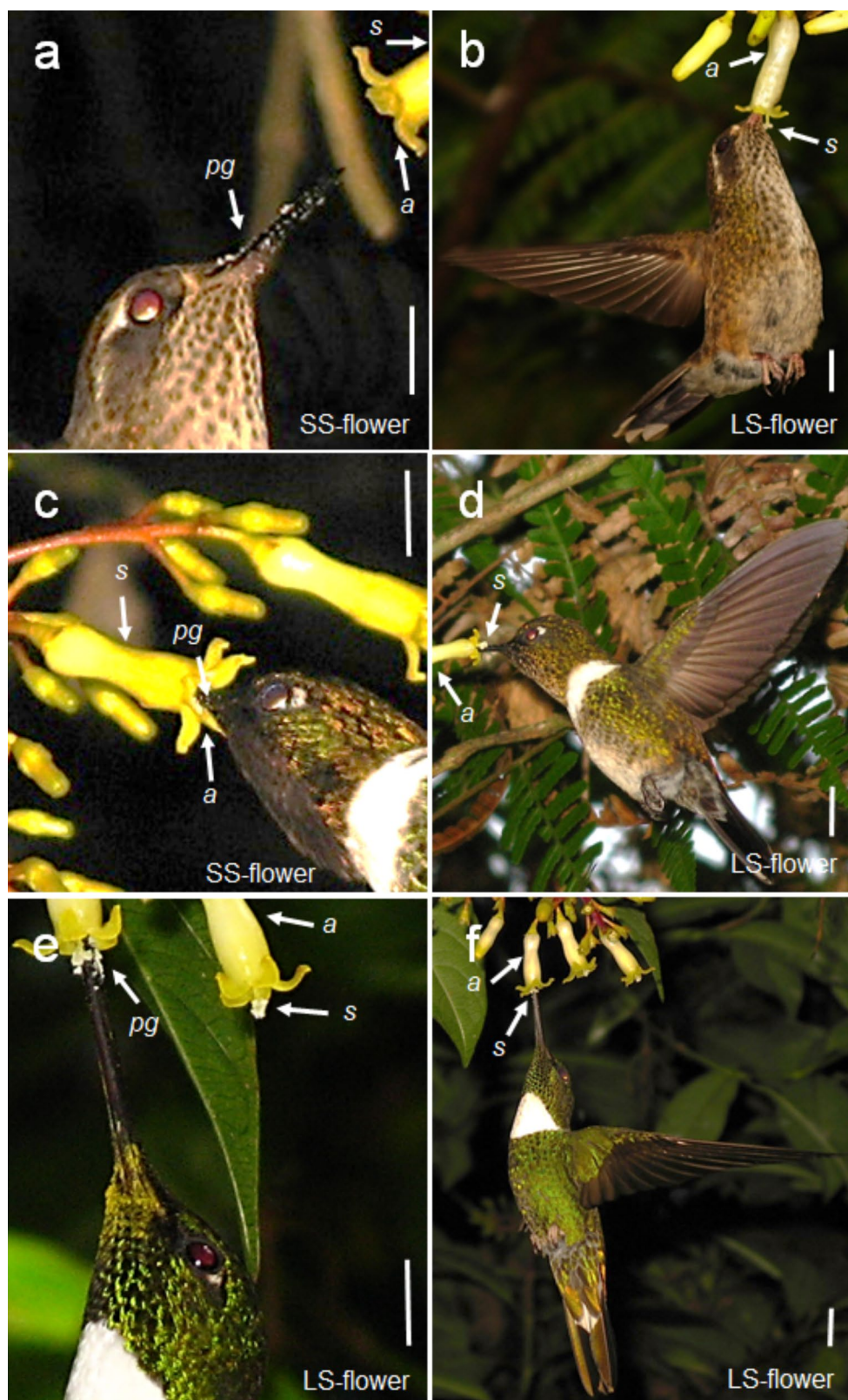
Table 1 Pollen loads deposited on SS-and LS-stigmas of *Palicouerea demissa* (Rubiaceae) after a single visit by hummingbird pollinators

Hummingbird pollinators (Bill size)	Type of pollen load	Stigma		U-test	
		SS	LS		
Coeligena torquata (33 mm)					
	Total	10.3 ± 6.2	5.7 ± 6.1	143	NS
	Alien	0.6 ± 0.8	1.1 ± 1.0	98.5	NS
	P. demissa	9.7 ± 6.1	4.6 ± 6.1	152	*
	Legitimate	7.5 ± 5.6 (LS)	3.9 ± 6.1 (SS)	138	NS
	Illegitimate	2.2 ± 1.5 (SS)	0.7 ± 1.1 (LS)	149	*
Heliangelus spencei (18 mm)					
	Total	8.9 ± 6.2	30.0 ± 22.4	64.0	**
	Alien	0.3 ± 0.7	2.5 ± 2.3	82.0	**
	P. demissa	8.5 ± 6.3	27.5 ± 22.2	66.0	*
	Legitimate	3.5 ± 4.9 (LS)	16.7 ± 11.6 (SS)	49.5	***
	Illegitimate	5.0 ± 3.3 (SS)	10.8 ± 11.0 (LS)	95.5	NS
Adelomia melanogenis (13 mm)					
	Total	20.3 ± 23.5	5.4 ± 3.0	119	*
	Alien	13.8 ± 21.5	0.7 ± 0.8	147	*
	P. demissa	6.5 ± 4.5	4.7 ± 3.1	107	NS
	Legitimate	3.1 ± 2.3 (LS)	3.7 ± 3.1 (SS)	109	NS
	Illegitimate	3.4 ± 3.3 (SS)	1.0 ± 1.5 (LS)	146	NS

Measurements are expressed in pollen grains per stigma and visit. Data indicate mean ± 2 SE. *NS* non-significant ($P > 0.05$), * $P = 0.05 < 0.09$ (marginally significant), ** $P < 0.05$, *** $P < 0.01$

species interaction was statistically significant ($F_{2,89} = 5.42$, $P = 0.006$, Table 1; Fig. 4).

When we evaluate the pollen load of *P. demissa* as proportions of legitimate and illegitimate pollen, there were no statistically significant differences between floral morphs (legitimate pollen, $F_{1,84} = 2.56$, $P = 0.11$; illegitimate pollen, $F_{1,84} = 2.39$, $P = 0.12$) or among hummingbird species (legitimate pollen, $F_{2,84} = 0.64$, $P = 0.53$; illegitimate pollen, $F_{2,84} = 2.70$, $P = 0.07$). However, the floral morph × hummingbird species effect was statistically significant for the proportion of legitimate pollen ($F_{2,89} = 4.45$, $P = 0.014$) but not for illegitimate pollen ($F_{2,84} = 0.15$, $P = 0.85$; Fig. 4), since *A. melanogenys* and *H. spencei* deposited proportionally more legitimate pollen on LS-stigmas while *C. torquata* deposited more legitimate pollen on SS-stigmas (Fig. 3). Similarly, the probability analyses of pollen transference between floral morphs showed that *H. spencei* and *A. melanogenys* have higher pollination probabilities from SS-anthers to LS-and SS-stigmas, and from LS-anther to LS-stigmas than from LS-anther to SS-stigmas. Contrarily,

C. torquata have higher probabilities of pollen transference from LS-anthers to SS-stigmas than in other directions. These trends are observed when comparing the relative importance of each pollen transfer probability promoted by each hummingbird species (Table 2).

Discussion

Asymmetric pollen flow and gender specialization

Hummingbirds play an important role as pollen vectors and pollinators in many distylous species, especially of Rubiaceae (De Castro and Araujo 2004; Murcia and Feinsinger 1996), as has been observed in *Palicourea* species (García-Robledo 2008; Morales et al. 2012; Ornelas et al. 2004a), including *P. demissa* (Valois-Cuesta and Novoa-Sheppard 2006; Valois-Cuesta et al. 2011b) and many other studies (See Cardoso et al. 2022 and studies cited there). Floral morphs of *P. demiss*a did not differ in the rate of visits by hummingbirds. However, SS- and LS-plants showed asymmetry in pollen flow, where SS-stigmas received more legitimate pollen than LS-stigmas; that is, SS-plants are more successful through their female sexual function while LS-plants are more successful in their male sexual function (Valois-Cuesta et al. 2011a, b, 2012). This indicates a possible transition towards gender specialization (Beach and Bawa 1980; Valois-Cuesta et al. 2012). The only caveat here is that we do not know exactly what was the pollen load on the hummingbird´s body before visiting focal flowers. A markedly different pollen load on the hummingbird's bill, with different legitimate vs. illegitimate pollen ratio, would affect compatible pollen deposition on stigmas (Maruyama et al. 2016; Raupp et al. 2020).

Pollen flow asymmetry between floral morphs of distylous species is common in Rubiaceae. Species such as *Arcytophyllum lavarum* K.Schum. (Garcia-Robledo 2008), *Mitchella repens* L. (Hicks et al. 1985), P. demissa (Valois-Cuesta et al. 2011b, 2012; this study), *Palicourea fendleri* Standl. (Lau and Bosque 2003), *Palicourea lasiorrachis* Oerst. (Feinsinger and Busby 1987), *Palicourea padifolia* (Willd. ex Schult.) C.M.Taylor & Lorence (Ornelas et al. 2004a), *Palicourea rigida* Kunth (Raupp et al. 2020), *Psychotria nuda* (Cham. & Schltdl.) Wawra (De Castro and Araujo 2004) and *Psychotria suerrensis* Donn.Sm. (Stone 1995) display asymmetric pollen transfer between morphs, in which, LS-morph is more efficient in reaching legitimate stigmas while SS-morph is more successful in the reception of legitimate pollen, even in different populations (Hernández and Ornelas 2007). Asymmetric pollen flow may promote gender specialization of each morph (Duan et al. 2018; Ornelas et al. 2020; Valois-Cuesta et al. 2012). Male and
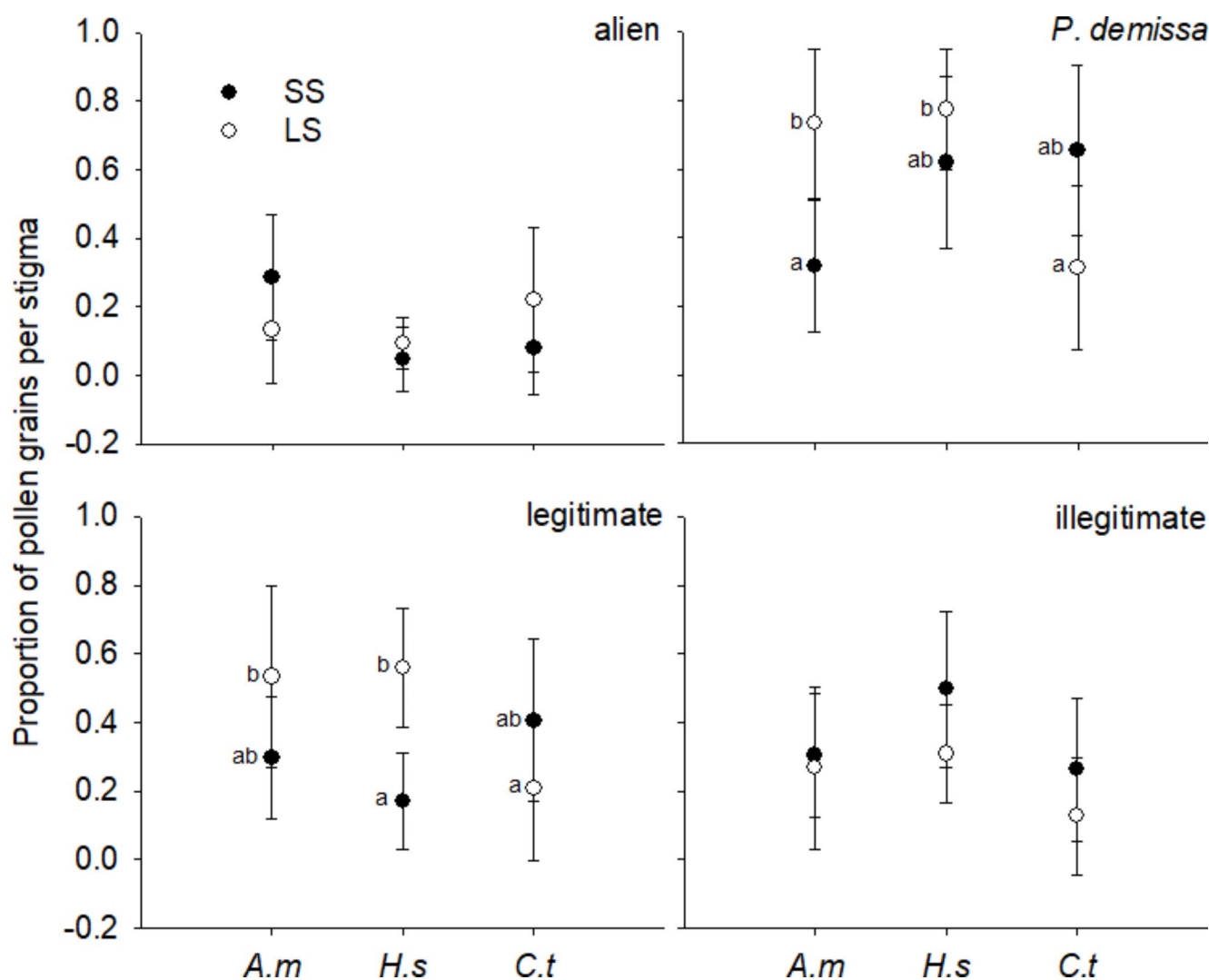
Fig. 4 Proportional pollen load deposited on stigmas by three hummingbird species after a single visit to SS- and LS-flowers of *Palicourea demissa* (Rubiaceae). *Coeligena torquata* (*C.t*), *Heliangelus* *spencei* (*H.s*) and *Adelomyia melanogenys* (*A.m*). Different letters indicate statistically significant differences (*P* < 0.01). Data indicate mean ± 2 SE.

female contributions to potential functional gender may be equivalent in both morphs. However, LS-morph may transmit more genes through pollen donation and SS-morph more through seed set (García-Robledo 2008; Valois-Cuesta et al. 2012). Distyly is the ancestral state in several dioecious species of Rubiaceae (Duan et al. 2018; Naiki and Kato 1999; Pailler et al. 1998). In a possible transition to dioecy from distyly, LS-morph presumably becomes male while SS-morph became female, reflecting the common pattern of asymmetry in pollen transport observed in *P. demissa*, in which, SS-morph receive more legitimate pollen and female fitness than LS-morph (Valois-Cuesta et al. 2011b, 2012; this study). Nevertheless, the dioecy has evolved from distyly in several species where SS-morph became male while LS-morph became female due to a possible change in the pollinator system, from pollinators of long-billed to short-billed pollinators that disrupted complementary pollen flow between morphs and promoted more legitimacy flow between more exposed sexual organs in flowers (Beach and Bawa 1980; Watanabe et al. 2014).

The ecological causes of the gender specialization in *P. demissa* (Valois-Cuesta et al. 2012) have not been fully explored but could include the way in that floral trail and pollinators interact (Barret 2002; Garcia-Robledo 2008; Hernández and Ornelas 2007; Morales et al. 2012; Murcia and Feinsinger 1996; Ornelas et al. 2004a; Raupp et al. 2020). Long- and short-billed hummingbirds may disrupt the complementarity of the legitimate pollen flow; thus, floral morphs may gradually specialize as either male or female (Beach and Bawa 1980; Duan et al. 2018; Watanabe et al. 2014).

Table 2 Probability of pollen deposition on a stigma Tij by hummingbird pollinators of *Palicourea demissa* (Rubiaceae)

Hummingbird pollinators (Bill size)	Pollen	Probability of a pollen grain being deposited on a stigma (RI %)	
		SS	LS
Coeligena torquata (33 mm)			
	SS	1.2×10^{-3} (15.6)	2.1×10^{-3} (27.3)
	LS	4.0×10^{-3} (51.9)	0.4×10^{-3} (5.19)
Heliangelus spencei (18 mm)			
	SS	2.7×10^{-3} (14.1)	8.9×10^{-3} (46.6)
	LS	1.8×10^{-3} (9.42)	5.7×10^{-3} (29.8)
Adelomia melanogenis (13 mm)			
	SS	1.8×10^{-3} (0.44)	2.0×10^{-3} (0.49)
	LS	1.6×10^{-3} (0.38)	0.5×10^{-3} (0.10)

Values in parentheses indicate the relative importance (RI %) of each pollen flow Tij

Long-and short-billed hummingbirds as pollen vectors

The effectiveness of legitimate pollen transfer between morphs by hummingbirds depends on the matching of their head morphology to the architecture of a plant's sexual structures (Murcia and Feinsinger 1996). Our data suggest that differences in legitimate pollen transfer between floral morphs of *P. demissa*, in which, SS-stigmas received proportionally more compatible pollen than LS-stigmas could be attributed to hummingbird visitation and its morphological fit to flowers of each morph (Murcia and Feinsinger 1996; Valois-Cuesta et al. 2011b, 2012). In *P. demissa*, SS-flowers display longer corolla tube, corolla-tube entrance width and stigmatic lobes than LS-flowers (Valois-Cuesta et al. 2011a; Fig. 1). Morph-differences in corolla-tube length, corolla-tube entrance width and stigmatic-lobes length in *Palicourea* species are often accompanied by asymmetric pollen flow, in which, SS-stigmas receive more compatible pollen than LS-stigmas (Feinsinger and Busby 1987; Hernández and Ornelas 2007; Lau and Bosque 2003; Ornelas et al. 2004a; Valois-Cuesta 2011a, 2012). The probability analysis of pollen transfer between morphs of *P. demissa* showed that *C. torquata* (long-billed hummingbird) is more effective for pollen transfer from LS-anthers to SS-stigmas (legitimate pollination) than in other directions, while *H. spencei* and *A. melanogenys* (short-billed hummingbirds) are more effective for pollen transfer from SS-anthers to LS-stigmas (legitimate pollination) and from LS-anthers to LS-stigmas (illegitimate pollination) than in any other possible combination (Table 2). The hummingbirds' effectiveness to promote male and female reproductive success of floral morphs may be explained because *C. torquata* with a long bill has a better chance to touch LS-anthers and SS-stigmas than short-bill hummingbirds. Unlike *H. spencei* y *A. melanogenys*, both with short bills, have a better chance of contact and pollen transfer between SS-anthers and LS-stigmas deployed in the corolla-tube entrance in both morphs (Fig. 3).

The lack of reciprocity in the position of sexual organs between morphs affects how pollen of each morph is deposited on the hummingbird's bill (Morales et al. 2012). In *Bouvardia ternifolia*, pollen from SS-flowers was mostly positioned in the proximal part of the bill (areas near the head) in short-billed hummingbirds, but pollen from LS-flowers was deposited in the distal and intermediate part of the bill in long-billed hummingbirds, indicating differential pollen deposition in the hummingbird's bill depends on the floral morph visited (Morales et al. 2012; see also Fig. 4). Similarly, Raupp et al. (2020) studied pollen deposition on hummingbird's bill by simulations of visits with a taxidermied specimen of *Thalurania glaucopisusing* (middle-billed hummingbird) and found that pollen from SS-flowers of *Palicourea rigida* was deposited at the base of the hummingbird's bill, while pollen from LS-anthers appeared on the distal and middle section of the bill. These researchers also found more legitimate pollen on SS than LS stigmas which was attributed to the higher reciprocity between LS-anthers and SS-stigmas (Raupp et al. 2020), as in *P. demissa* (Valois-Cuesta 2011a, b). As opposed to this trend, the reciprocity was higher between SS-anther and LS-stigma in *Jasminum malabaricum*, but the proportion of legitimate pollen was higher on SS than LS-stigmas (Ganguly and Barua 2021), suggesting that reciprocal herkogamy alone does not explain patterns of legitimate pollen deposition on stigmas of both morphs after hummingbird visits (Trevizan et al. 2021).

Although reciprocal herkogamy has been interpreted as a mechanism that facilitates cross-pollination (Barret 2002), other floral traits affect pollen deposition on stigmas in distylous, hummingbird-pollinated species (Hernández and Ornelas 2007; Trevizan et al. 2021). Some works show that differences in corolla-tube length promote differential pollinator visitation and differential pollen donation and receipt (Nishihiro et al. 2000; Ornelas et al. 2004a, b; Wolfe and Barrett 1989). Differences in corolla-tube entrance width may determine the effectiveness of hummingbirds as pollinators; for example, legitimate pollination decreased with increased width of corolla entrance in LS-flowers while the effect was positive in SS-flowers of *P. padifolia* (Hernández and Ornelas 2007). Stigma-size dimorphism has been interpreted as a mechanism to increase the area of pollen reception in flowers with less accessible stigmas, enhancing the

nature of stigmatic pollen loads, and the relative reproductive fitness of a particular morph (Dulberger 1992; Ornelas et al. 2004a). There is a correlation between stigmatic lobe length and legitimate pollen deposition, with longer stigmatic lobes presenting proportionally more legitimate pollen (Furtado et al. 2021).

On the other hand, pollinator visitation has a direct, positive, linear effect on pollen receipt; that is, an increase in pollinator visitation is translated in most pollen receipt and seed sets (Engel and Irvin 2003). In *P. demissa*, the hummingbird *C. torquata* (SS $= 1.44 \pm 0.67$; LS $= 1.67 \pm 0.72$) has higher visit rate (visits plant hour^{-1}) than *H. spencei* (SS $= 0.72 \pm 0.40$; LS $= 1.24 \pm 0.57$) and *A. melanogenys* (SS $= 1.16 \pm 0.43$; LS $= 0.64 \pm 0.43$) (Valois-Cuesta and Novoa-Sheppard 2006; Valois-Cuesta et al. 2011b). Thus, a higher probability of pollen transfer from LS to SS-morph mediated by *C. torquata* and its higher visit rate vs. *H. specie* and *A. melanogenys*, may explain the higher female success of SS- than LS-morph in *P. demissa* (Valois-Cuesta et al. 2011b, 2012, this study). It is also possible that greater female reproductive success of SS-morph is due to loss of male fertility through abnormalities in pollen development as has been observed in other *Palicourea* species (Ornelas et al. 2020). In this study, the role of less frequent floral visitors was not evaluated. However, we recognize that studying all guild members of floral visitors may help explain observed patterns of male and female reproductive success between SS- and LS-plants.

Overall, floral morphs of *P. demissa* display imperfect reciprocal herkogamy and differ in other morphological traits such as corolla and stigma size, which affects how legitimate pollen is deposited on stigmas of each morph by long- and short-billed hummingbird. This differential deposition of legitimate pollen by hummingbird species may have consequences on the sexual expression of each morph (SS-morph is more female than LS-morph) since long-billed hummingbirds are better pollinators of SS-stigmas, while short-billed hummingbirds tend to deposit both legitimate and illegitimate pollen on SS-and LS-stigmas. Nevertheless, a better understanding of the sexual expression of SS- and LS-plants will depend on future experimental research that involves possible morpho-differences in the biological function of male and female floral structures of each morph, as well as the role played by less frequent floral visitors (insects) as pollen vectors and pollinators of floral morphs of *P. demissa*.

Declarations

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