

# Appendix S1: Testing for pollinator recognition in multiple species of *Heliconia*

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## **1 Supplemental methods**

### **2 Aviary experiments**

3 In identifying focal *Heliconia* species, we first identified species which were likely to have multiple individual  
4 plants (rather than multiple clones) in the Las Cruces Biological Station living collection and the surrounding  
5 area based on collection records and surveys of the area. Because *Heliconia* are self-incompatible to partially  
6 self-compatible (W. J. Kress 1983; Pedersen and Kress 1999; Janeček et al. 2020; Betts, Hadley, and Kress  
7 2015), we sought to maximize the genetic diversity of the pollen pool in order to limit the possibility of  
8 failing to detect a difference among pollination treatments simply due to a lack of compatible pollen. As  
9 an additional confirmation that compatible pollen was available in the area, we required that plants could  
10 be seen setting fruit. Indeed, these measures did not eliminate the possibility of non-detection due to poor  
11 quality pollen since we do not know the parentage of the plants used in the experiments, but this should  
12 not inflate the chances of erroneously detecting pollinator recognition since pollen quality was held constant  
13 across control flowers and treatment flowers.

14 Prior to anthesis, all flowers were covered with a mesh bag in order to preclude visits from free ranging  
15 pollinators. Each flower was hand pollinated by the same experimenter (author D.G. Gannon), using pollen  
16 sourced from plants located at least five meters away from the focal plant in order to reduce the chances of  
17 applying self or related pollen to the stigma (mean distance to donor  $\pm$  1 sd:  $\bar{x} = 233\text{m} \pm 223\text{m}$ ). We gently  
18 separated styles from the stamens using forceps and cleaned all pollen from the stigma with a cotton swab  
19 under 20x magnification. We then adhered pollen to the stigmatic surface by scraping the pollen from an  
20 anther of the donor flower with a toothpick and touching the toothpick to the stigma of the focal flower. We  
21 checked that pollen adhered to the stigmatic surface and that pollen was dispersed across the stigma in a  
22 relatively even layer using a 20x hand lens. As mentioned in the main text, quantification of pollen grains

23 on the stigma in the field is not feasible due to the size of the pollen grains, but we attempted to minimize  
24 variation in the quantity of pollen applied across treatments and replicates.

25 Aviary experiments began by locating a bagged inflorescence with two mature flowers. We hand-pollinated  
26 each flower using the methods described above, then assigned flowers to a treatment at random, one flower  
27 assigned as a control (hand-pollination only) and the other to be visited by a hummingbird (long-billed or  
28 short-billed). The control flower was covered with a red paper sleeve to block access. We erected small,  
29 portable aviaries (1m x 1m x 2m) to enclose focal plants (Figure S1). Aviaries were constructed from sewn  
30 shade cloth, a one-inch PVC hoop at the top, and bamboo legs that could be embedded in the ground.

31 We used two common hummingbird species in our aviary experiments which represent guilds of short-  
32 billed and long-billed hummingbirds: rufous-tailed hummingbirds (*Amazilia tzacatl*), and green hermit  
33 hummingbirds (*Phaethornis guy*), respectively (Figure 1, Main text). We captured hummingbirds using  
34 standard mist-netting procedures (OSU ACUP 5020), running nets from 0600 to 1000 hours. Males of focal  
35 bird species were placed in cloth bags for transport to the aviary, and all non-target species were immediately  
36 released. On some occasions, male hummingbirds birds were housed in a 2 m × 2 m × 1.5 m aviary for up to  
37 seven days to facilitate data collection (OSU ACUP 5020). This became necessary as capture rates decreased  
38 in the area of the experiments.

39 To ensure that hummingbirds did not contribute additional pollen to the stigmatic surface of the focal  
40 flower, we cleaned hummingbirds of all pollen under 20x magnification using damp cotton swabs and a  
41 photographer's brush before releasing them into the aviary. Hummingbirds were provided a perch inside  
42 the aviary and supplemental sugar water (20% sucrose by mass) if the hummingbird did not visit the focal  
43 flower after 30 minutes post-entry. At 60 minutes, if the hummingbird had not visited the focal flower,  
44 we terminated the experiment and the bird was either released or fed sugar water and moved to another  
45 experiment. We conducted all pollination experiments between the hours of 0600 and 1100, as *Heliconia*  
46 pollen viability and/or stigmatic receptivity may be in question later in the day (Dafni and Firmage 2000;  
47 Heddly, Hormaza, and Herrero 2003; Schleuning et al. 2011). Flowers were labeled, covered with mesh bags  
48 to ensure no additional pollinator visits, and collected the following day after abscission.

#### 49 Nectar removal experiments

50 To test whether nectar removal may provide a mechanism conferring pollinator recognition in *Heliconia*, we  
51 manually extracted nectar from flowers of three of the six focal species based on flower availability. We again  
52 included at least one control flower for each day and for each species on which conducted the experiments. We  
53 extracted nectar from flowers using a 20 µL micropipette tip bent to match the curvature of the flower. To  
54 the back of the pipette tip, we fit a length of 0.5 mm plastic tubing, connected to a 20 mL syringe with which



Figure S1: Portable aviary assembled around a live inflorescence.

55 we created suction. We measured the volume of liquid removed with a glass capillary tube, then dispensed it  
 56 onto a temperature-calibrated hand refractometer to verify that the liquid was likely nectar produced by the  
 57 flower. The minimum Brix index observed in an independent dataset of *Heliconia* nectar was c.a. 14.1% (K.  
 58 Leimberger *unpublished data*). Thus, we recorded instances in which the sugar concentration measured below  
 59 this level.

60 An alternative stimulus to which plants could respond is the mechanical action of a hummingbird inserting  
 61 its bill into the perianth to access the nectar. To test whether we could induce pollen tube growth by the  
 62 mechanical stimulus of inserting a morphologically matched object into the flower, we conducted experiments  
 63 in which we simply inserted the pipette tip, then removed it without extracting any nectar. Nectar volumes  
 64 moving up into the pipette tip due to capillary action were, except for in a few cases, not measurable. This  
 65 experiment is therefore not confounded with the nectar removal experiments.

66 Indeed, our nectar removal treatments could not completely mimic the way in which birds interact with a  
 67 flower, since pollinators deposit pollen at the same time nectar is removed. In our experiments, we could only  
 68 complete these tasks in sequence. We therefore conducted some nectar removal experiments in which we  
 69 hand pollinated the flower after removing nectar. These experiments allowed us to assess whether timing of  
 70 nectar removal relative to pollen deposition could be important to pollen germination success.

71 **Laboratory methods**

72 We collected styles the following day (after abscission) and fixed them in formalin acetyl acid for at least  
 73 72 hours before transferring them to 70% ethanol for transport. We stained pollen tubes using aniline blue  
 74 dye in a buffer of KHPO<sub>4</sub> preceded by four wash steps: 24 hours in dH<sub>2</sub>O, 24 hours in 5M NaOH to soften  
 75 the tissues, followed by two 24-hour dH<sub>2</sub>O washes (Kress 1983; Betts, Hadley, and Kress 2015). Styles were  
 76 mounted on microscope slides and scored for pollen tube rates by counting the maximum number of pollen  
 77 tubes found in any given cross-section of the style using an epi-fluorescence microscope.

78 **Trait (mis)matching**

79 Upon capturing hummingbirds, we photographed their bills against a 0.25-inch gridded notebook. We then  
 80 used ImageJ to measure the length of the bill along the outer (top) surface of the bill using the ‘line segment’  
 81 feature after setting the scale using the gridded paper. To measure curvature, we utilized the methods of  
 82 Temeles et al. (2009) in which we related the outer edge of the bill to a circle (Figure S2). The curvature of a  
 83 circle is measured as the inverse of its radius. To estimate the radius of a circle with the same curvature as a  
 84 bird’s bill, we connected two points on the curved section of the bill, effectively drawing a chord on the circle  
 85 (Figure S2). We then measured the angle between the chord and the line tangent (labeled *T*) to the bill at  
 86 one end of the chord, known as the *angle of declension* ( $\theta$  on Figure S2). Straight-forward trigonometric  
 87 identities then yield  $\hat{r} = \frac{C/2}{\sin \theta}$ , where *C* is the length of the chord (mm) and  $\theta$  is the angle of declension  
 88 (radians). Thus, we use  $\hat{r}^{-1}$  as an estimate of the curvature of a bill or flower.

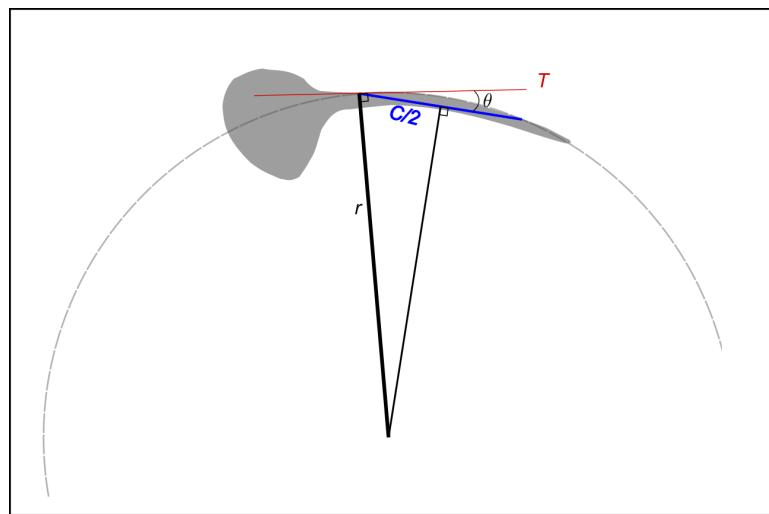


Figure S2: Relating the arc of the top of a bird’s bill to a circle as in Temeles et al. (2009).

89 **Statistical methods**

90 Let  $y_{ijkl} \in \mathbb{N}^+$  be the number of pollen tubes scored in the  $l^{\text{th}}$  flower from the  $k^{\text{th}}$  plant of species  $j = 1, \dots, s$ ,  
 91 and let  $i$  index the treatment ( $i = 1, \dots, g$ ). We assume that  $y_{ijkl} \stackrel{iid}{\sim} \text{Poisson}(\lambda_{ijk})$  for  $l = 1, \dots, n_k$ . The model  
 92 for the experiment can be written as

$$\log(\lambda_{ijk}) = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_{k(j)}$$

93 where

- 94 •  $\mu$  is the overall mean log-pollen tube rate,
- 95 •  $\alpha_i$ ,  $\sum_{i=1}^g \alpha_i = 0$ , is the average deviation from the mean for flowers that received treatment  $i$ ,
- 96 •  $\beta_j$ ,  $\sum_{j=1}^s \beta_j = 0$ , is the average deviation from the mean for flowers of plant species  $j$ ,
- 97 •  $(\alpha\beta)_{ij}$ ,  $\sum_{i=1}^g (\alpha\beta)_{ij} = \sum_{j=1}^s (\alpha\beta)_{ij} = 0$ , is the species  $\times$  treatment interaction effect,
- 98 •  $\gamma_{k(j)} \stackrel{iid}{\sim} \mathcal{N}(0, \sigma_j^2)$ , for the  $k = 1, \dots, p_j$  plants of species  $j$ , is a random effect for the plant that is nested  
 99 within species.

100 Because this model has an unconventional variance structure (random effects have unique variances  
 101 depending on the plant species), we fit this model in a Bayesian framework. Furthermore, for ease of  
 102 defining the model in `rstan` (Carpenter et al. 2017), we reparameterized the model to follow a regression  
 103 parameterization (reference level for the aviary experiments was hand pollination for *H. hirsuta* and hand  
 104 pollination for *H. rostrata* for the nectar removal experiments). The model therefore reads

$$\log \boldsymbol{\lambda} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\boldsymbol{\gamma},$$

105 where  $\mathbf{X}$  is a matrix of indicator variables indicating the species and treatment and  $\mathbf{Z}$  is a matrix of  
 106 indicator variables indicating the plant individual for the  $i^{\text{th}}$  observation,  $i = 1, \dots, n$ .

107 **Priors**

108 We use weakly informative priors (priors designed in a such a way as to be intentionally less-informative than  
 109 a prior reflecting the information actually available (Gelman 2006) for all regression coefficients such that  
 110 all are independent and normally distributed with mean zero and standard deviation 1.5 (on the log scale).  
 111 Importantly, a standard deviation of 1.5 was chosen to constrain samplers to biologically reasonable parameter  
 112 space based on available literature reporting pollen tube rates in Heliconia. W. J. Kress (1983) reports a

113 ~4.08-fold change (a regression coefficient of ~1.407), comparing minimum and maximum pollen tube rates  
114 from nine species. Similarly, (Pedersen and Kress 1999) report a ~4.00-fold increase in pollen tube rates in  
115 H. paka when comparing flowers visited by honeyeaters to cross-pollinations by hand, a ~60-fold increase  
116 comparing rates of autogamous selfing to honeyeater pollination, and a 3.3-fold increase in pollen tube rates  
117 in H. laufo when comparing autogamous selfing to honeyeater pollination. Previous work in this system  
118 shows pollen tube rates in H. tortuosa are maximized following visits from violet sabrewing hummingbirds  
119 (Campyloterus hemileucurus), a ~5.7-fold increase from ineffective pollinators (Betts, Hadley, and Kress 2015).  
120 A  $\mathcal{N}(0, 1.5)$  prior on regression coefficients on the log scale reflects the presumption that most (95%) of the  
121 time, we will not see fold-changes greater than ~20 when comparing pollen tube rates in Heliconia. Thus,  
122 we think these priors are a good balance among being conservative (concentrating some mass around zero  
123 such that signals in the data need to be strong to suggest differences between treatments), vague about prior  
124 information, and within the realm of biological plausibility.

125 For the species-specific scale parameters, we define the priors  $\sigma_j \sim \text{half-Normal}(0, 1)$  for each  $j \in \{1, 2, 3, 4\}$ .  
126 This structure allows us to account for potential correlation among flowers from the same individual plant  
127 without assuming all plant effects are i.i.d. random variables, but instead that plant effects are i.i.d. within a  
128 species.

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