

Testing for pollinator recognition in multiple species of *Heliconia*:

Supplementary material

D.G. Gannon, A.S. Hadley, U.G. Kormann, F.A. Jones, M.G. Betts

Contents

Experimental methods	1
Aviary experiments	1
Nectar removal experiments	2
Laboratory methods	4
Trait (mis)matching	4
Statistical methods	4
Alternative mechanisms of recognition	6
References	6

1 Experimental 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
17 of 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
18 gently separated styles from the stamens using forceps and cleaned all pollen from the stigma with a cotton
19 swab under 20x magnification. We then adhered pollen to the stigmatic surface by scraping the pollen from
20 an anther of the donor flower with a toothpick and touching the toothpick to the stigma of the focal flower.
21 We checked that pollen adhered to the stigmatic surface and that pollen was dispersed across the stigma in
22 a 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 ($1\text{m} \times 1\text{m} \times 2\text{m}$) to enclose focal plants (Fig 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 hum-
33 mingbirds (*Phaethornis guy*), respectively (Fig 1, main text). We captured hummingbirds using standard
34 mist-netting procedures (OSU ACUP 5020), running nets from 0600 to 1000 hours. Males of focal bird
35 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 $2\text{ m} \times 2\text{ m} \times 1.5\text{ m}$ aviary for
37 up to seven days to facilitate data collection (OSU ACUP 5020). This became necessary as capture rates
38 decreased 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 Hedhly, 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.

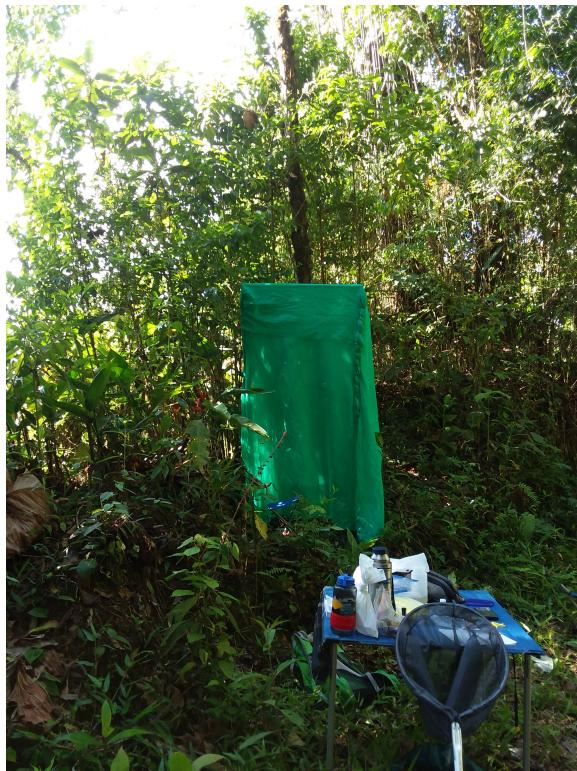


Figure S1: Portable aviary assembled around a live inflorescence.

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
55 we created suction. We measured the volume of liquid removed with a glass capillary tube, then dispensed
56 it onto a temperature-calibrated hand refractometer to verify that the liquid was likely nectar produced by
57 the flower. The minimum Brix index observed in an independent dataset of *Heliconia* nectar was c.a. 14.1%

58 (K. Leimberger *unpublished data*). Thus, we recorded instances in which the sugar concentration measured
59 below 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
67 a flower, since pollinators deposit pollen at the same time nectar is removed. In our experiments, we could
68 only complete these tasks in sequence. We therefore conducted some nectar removal experiments in which
69 we hand pollinated the flower after removing nectar. These experiments allowed us to assess whether timing
70 of 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 72
73 hours before transferring them to 70% ethanol for transport back to Oregon State University. We stained
74 pollen tubes using aniline blue dye in a buffer of KHPO₄ preceded by four wash steps: 24 hours in dH₂O, 24
75 hours in 5M NaOH to soften the tissues, followed by two 24-hour dH₂O washes (Kress 1983; Betts, Hadley,
76 and Kress 2015). We mounted styles on microscope slides and scored pollen tubes by counting the maximum
77 number of pollen 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
83 a circle is measured as the inverse of its radius. To estimate the radius of a circle with the same curvature
84 as a bird’s bill, we connected two points on the curved section of the bill, effectively drawing a chord on the
85 circle (Figure S2). We then measured the angle between the chord and the line tangent (labeled *T*) to the
86 bill at one end of the chord, known as the *angle of declension* (θ in Fig 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 θ is the angle of declension
 88 (radians). Thus, we use \hat{r}^{-1} as an estimate of the curvature of a bill or flower.

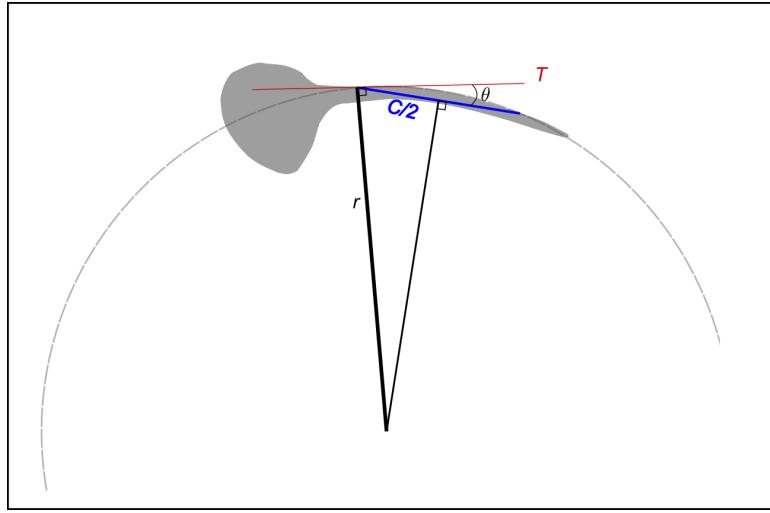


Figure S2: Relating the arc of the top of a bird's bill to a circle as in Temeles et al. (2009).

89 Statistical methods

90 Let $y_{ijkl} \in \mathbb{N}$ be the number of pollen tubes scored in the l^{th} flower from the k^{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
 92 model for the experiment can be written as

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

93 where

- 94 • μ is the overall mean log-pollen tube rate,
- 95 • α_i , $\sum_{i=1}^g \alpha_i = 0$, is the average deviation from the mean for flowers that received treatment i ,
- 96 • β_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 • $\gamma_{1(1)}, \gamma_{2(1)}, \dots, \gamma_{K_1(1)}, \gamma_{1(2)}, \dots, \gamma_{K_s(s)} \stackrel{iid}{\sim} \mathcal{N}(0, \sigma_\gamma^2)$, for the $k = 1, \dots, K_j$ plants of species j and all species
 99 $j = 1, 2, \dots, s$ are random effects for the plants that are nested within species.

100 For ease of defining the model in the R package `lme4` (Bates et al. 2015), we reparameterized the model
 101 to follow a regression parameterization (reference level for the aviary experiments was hand pollination for

¹⁰² *H. hirsuta* and hand pollination for *H. rostrata* for the nectar removal experiments). The model therefore
¹⁰³ reads

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

¹⁰⁴ where \mathbf{X} is a matrix of indicator variables indicating the species and treatment and \mathbf{Z} is a matrix of indicator
¹⁰⁵ variables indicating the plant individual for the i^{th} observation, $i = 1, \dots, n$.

¹⁰⁶ Alternative mechanisms of recognition

¹⁰⁷ We have considered a number of alternative hypotheses regarding the mechanism of recognition during our
¹⁰⁸ time studying *Heliconia* plants. Aside from differential nectar removal by different floral visitors, these include
¹⁰⁹ sonic cues provided by the beating of a hummingbird's wings (similar to what Veits et al. (2019) found with
¹¹⁰ nectar production in *Oenothera drummondii* plants), mechanical stimuli that cannot be reproduced using
¹¹¹ a pipette tip (Braam 2005), electrostatic interactions between pollen and negatively charged feathers of
¹¹² hummingbirds (Badger et al. 2015), and a reduction in self-pollen interference after a morphologically
¹¹³ matched hummingbird removes pollen. Future experiments that test all of these alternative mechanisms are
¹¹⁴ warranted.

¹¹⁵ Currently, we have some evidence to support the hypothesis that pollen removal by morphologically
¹¹⁶ matched hummingbirds could reduce self-pollen interference. For a small number of single aviary visits,
¹¹⁷ we collected pollen from birds' heads and bills following the visit. Green Hermits removed 1,221.8 pollen
¹¹⁸ grains from the flowers of *H. tortuosa* plants on average ($n = 10$ visits, $s_x = 695.67$ grains, where s_x denotes
¹¹⁹ the sample standard deviation) and Rufous-tails removed 202 grains on average ($n = 3$ visits, $s_x = 163.37$
¹²⁰ grains). This suggests that morphologically matched hummingbirds could reduce self-pollen interference
¹²¹ at the stigmatic surface to a greater degree than mismatched pollinators would. Furthermore, the result
¹²² that hand-pollinating after manual nectar removal produced the fewest numbers of pollen tubes per style of
¹²³ any treatments (Fig 3, main text) supports that reducing pollen interference at the stigmatic surface could
¹²⁴ promote pollen germination and tube growth. However, prior experiments with a hummingbird cadaver,
¹²⁵ which should remove an equivalent amount of pollen from the flower as a live bird would, did not stimulate
¹²⁶ pollen tube growth. Thus, this hypothesis requires further investigation.

127 References

- 128 Badger, Marc, Victor Manuel Ortega-Jimenez, Lisa von Rabenau, Ashley Smiley, and Robert Dudley. 2015.
 129 “Electrostatic Charge on Flying Hummingbirds and Its Potential Role in Pollination.” *PLOS ONE* 10 (9):
 130 e0138003. <https://doi.org/10.1371/journal.pone.0138003>.
- 131 Bates, Douglas, Martin Machler, Ben Bolker, and Steve Walker. 2015. “Fitting Linear Mixed-Effects
 132 Models Using Lme4.” *Journal of Statistical Software* 67 (1): 1–48. <https://doi.org/10.18637/jss.v067.i01>.
- 133 Betts, Matthew G., Adam S. Hadley, and W. John Kress. 2015. “Pollinator Recognition by a Keystone
 134 Tropical Plant.” *Proceedings of the National Academy of Sciences* 112 (11): 3433–8. <https://doi.org/10.1073/pnas.1419522112>.
- 135 Braam, Janet. 2005. “In Touch: Plant Responses to Mechanical Stimuli.” *New Phytologist* 165 (2):
 136 373–89. <https://doi.org/10.1111/j.1469-8137.2004.01263.x>.
- 137 Dafni, A., and D. Firmage. 2000. “Pollen Viability and Longevity: Practical, Ecological and Evolutionary
 138 Implications.” *Plant Systematics and Evolution* 222 (1-4): 113–32. <https://doi.org/10.1007/BF00984098>.
- 139 Hedhly, A., J. I. Hormaza, and M. Herrero. 2003. “The Effect of Temperature on Stigmatic Receptivity
 140 in Sweet Cherry (*Prunus Avium L.*).” *Plant, Cell & Environment* 26 (10): 1673–80. <https://doi.org/10.1046/j.1365-3040.2003.01085.x>.
- 141 Janeček, Štěpán, Kryštof Chmel, Guillermo Uceda Gómez, Petra Janečková, Eliška Chmelová, Zuzana
 142 Sejfová, and Francis Luma Ewome. 2020. “Ecological Fitting Is a Sufficient Driver of Tight Interactions
 143 Between Sunbirds and Ornithophilous Plants.” *Ecology and Evolution* 10 (4): 1784–93. <https://doi.org/10.1002/ece3.5942>.
- 144 Kress, W. J. 1983. “Crossability Barriers in Neotropical Heliconia.” *Annals of Botany* 52 (2): 131–47.
- 145 Kress, W. John. 1983. “Self-Incompatibility in Central American Heliconia.” *Evolution* 37 (4): 735–44.
 146 <https://doi.org/10.2307/2407915>.
- 147 Pedersen, Louise B., and W. John Kress. 1999. “Honeyeater (Meliphagidae) Pollination and the Floral
 148 Biology of Polynesian Heliconia (Heliconiaceae).” *Plant Systematics and Evolution* 216 (1): 1–21. <https://doi.org/10.1007/BF00985098>.
- 149 Schleuning, Matthias, Mathias Templin, Vicky Huamán, Giovana P. Vadillo, Thomas Becker, Walter
 150 Durka, Markus Fischer, and Diethart Matthies. 2011. “Effects of Inbreeding, Outbreeding, and Supplemental
 151 Pollen on the Reproduction of a Hummingbird-Pollinated Clonal Amazonian Herb.” *Biotropica* 43 (2): 183–
 152 91. <https://doi.org/10.1111/j.1744-7429.2010.00663.x>.
- 153 Temeles, Ethan J., Carolyn R. Koulouris, Sarah E. Sander, and W. John Kress. 2009. “Effect of Flower
 154 Shape and Size on Foraging Performance and Trade-Offs in a Tropical Hummingbird.” *Ecology* 90 (5):

159 1147–61. <https://doi.org/10.1890/08-0695.1>.

160 Veits, Marine, Itzhak Khait, Uri Obolski, Eyal Zinger, Arjan Boonman, Aya Goldshtain, Kfir Saban, et
161 al. 2019. “Flowers Respond to Pollinator Sound Within Minutes by Increasing Nectar Sugar Concentration.”

162 *Ecology Letters* 0 (0). <https://doi.org/10.1111/ele.13331>.