Testing for pollinator recognition in multiple species of *Heliconia*

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Abstract

Plant-pollinator generalization increases the robustness of plant-pollinator communities to fluctuations in the availability of mutualistic partners. However, natural selection may reinforce floral traits that filter pollinator communities to promote pollination by efficient pollinators, which reduces the number of potential partners. We tested the generality of a recently described, cryptic pollinator filter termed 'pollinator recognition' (PR) which could reduce the realized number of pollinators compared to the number of floral visitors. PR was first documented experimentally in *Heliconia tortuosa* whereby pollen tube germination – a proxy for reproduction – was enhanced following visits from morphologically specialized pollinators, but not generalists. We tested for PR in four taxa spread widely across the Heliconiaceae.

With aviary experiments that standardized pollen quality and minimized variation in pollen quantity, we demonstrated that visits by pollen-free hummingbirds increased pollen tube rates compared to hand pollination alone in two species; we also corroborated previous results that visits by long-billed, but not short-billed hummingbirds increased pollen tubes in *H. tortuosa*. Based on these results, we suggest that the PR mechanism likely exists beyond a single species in the Heliconiaceae and could promote outcrossing in two ways. First, promoting pollen tube growth following a cue from a visiting pollinator could allow a plant to invest in seed production when pollen on the stigma is less likely to be composed entirely of self pollen. Second, because hummingbirds with long, decurved bills tend to be less territorial and more mobile than those with short, straight bills, pollinator recognition could act in conjunction with physical exploitation barriers, such as long and curved corolla tubes, to filter the community of floral visitors in favor of those that are more likely to carry pollen from geographically and therefore genetically distance sources. While the mechanism underlying PR remains equivocal,

cryptic pollinator filters may be more common than previously anticipated and could alter perspectives on redundancy in plant-pollinator communities.

Introduction

Generalized pollination systems in which plants are pollinated by many floral visitors, each of which may visit many plants, are expected to result in communities that are more robust to fluctuations in the abundance of mutualistic partners [1–6]. This stability is derived through redundant interactions and may be important to the maintenance of biodiversity in plant-pollinator communities during an era of global climate change and anthropogenic disturbances [4,7,8]. However, natural selection may favor floral traits that filter the community of floral visitors to promote pollination by efficient mutualistic partners [9–12].

Pollinator filters, in general, manipulate animal visitation patterns. For example, nectar that is distasteful to some pollinators will deter them from visiting [13] and exploitation barriers, such as long corolla tubes that limit access to floral rewards [14,15], may make visitation unprofitable for some animals [16,17]. These filters reduce the generality of visitation patterns so their effects should be evident in observations of species interactions (generally pollinator visits to flowers); however, previous work with *Heliconia tortuosa* (Heliconiaceae) documented a cryptic pollinator filter that promotes pollen germination and pollen tube growth based on the identity, behavior, and bill morphology of hummingbird floral visitors [18].

In single-visit aviary experiments that controlled for variation in pollen deposition and visitation rates by different pollinator species [18], the number of pollen tubes that germinated in a style (henceforth ‘pollen tube rate’) was nearly six times greater in flowers visited by hummingbirds with bill shapes that are morphologically matched to the flowers (i.e., long,

decurved bills) than in flowers visited by hummingbirds with mismatched bill shapes. Furthermore, in a separate experiment, manual nectar removal showed higher pollen tube rates than hand pollination alone. Betts et al. coined this behavior 'pollinator recognition' and posited that nectar removal and pollen deposition by long-billed hummingbirds provides a cue for pollen grain germination and pollen tube growth, thus reducing pollination efficiency by morphologically mismatched hummingbirds that visit and transfer pollen but cannot access the full volume of nectar at the base of the flower [18].

Betts et al. (18) speculated that pollinator recognition may be adaptive if it allows plants to invest in reproduction following a visit from a pollinator that is more likely to carry high-quality pollen and limit reproduction with the pollen deposited by a pollinator more likely to carry pollen of low quality. Despite receiving visits from at least six hummingbird species, *H. tortuosa* specializes on long-billed hummingbirds that are highly mobile [18,19] compared to the short-billed hummingbirds which tend to defend territories and therefore move less. The mobile foraging behaviors of these birds may make them more likely to carry high-quality pollen from geographically and genetically distant sources [20]. Indeed, recent work supports that pollination by large-bodied, traplining hummingbirds with long, decurved bills promotes outcrossing among *H. tortuosa* individuals [21].

We postulated that pollinator recognition may occur in other plant taxa, particularly in relatively stable tropical systems with high pollinator diversity. Determining whether this is the case is important for two reasons. First, pollinator recognition could be one means through which tight morphological matching evolves despite apparently generalized interaction networks. Second, if the number of realized pollinator species is much smaller than the number of observed floral visitors, pollination networks could be more vulnerable to loss of specialist pollinators

under anthropogenic change [4,5]. We tested for pollinator recognition in four species distributed widely across the Heliconiaceae phylogeny.

Materials and Methods

Study species

Heliconiaceae is a monogeneric family consisting of an estimated 200-250 species which radiated rapidly c.a. 39-24 million years ago [22]. *Heliconia* species are rhizomatous perennial herbs distributed widely throughout the Neotropics and on some South Pacific islands. Flowers are situated within showy bracts and composed of six tepals, five of which are fused to create a cylindrical perianth, the sixth peels back upon anthesis. A defining feature of the Heliconiaceae is a staminode (modified stamen) that partially covers the opening to the nectar chamber at the base of the perianth, which may need to be moved by a visiting animal when they extract the nectar reward (though the mechanics of this have not be studied in detail). Flowers of the Heliconiaceae last a single day from anthesis to dehiscence.

We targeted species that were common in the living collection at the Organization of Tropical Studies Las Cruces Biological Station, Puntarenas Province, Coto Brus, Costa Rica, (8° 47′ 7″ N, 82° 57′ 32″ W) and could be found naturally or in ornamental gardens in the area. We required that plants were setting seed when left unmanipulated, indicating that a viable pollen source existed in the area, since previous work on mating systems in *Heliconia* suggests that the hermaphroditic flowers of many species are self-incompatible to partially self-compatible, but largely not selfing [18,21,23–26]. Furthermore, we required that wild, native hummingbirds could be seen visiting the flowers of each target species in camera trap data [27] or during observation, indicating that wild-caught hummingbirds would visit and drink from the flowers

inside aviaries despite the fact that many plant species in the collection are not native to Costa Rica. The plant species that met these criteria included *H. hirsuta*, which is native to South America and Trinidad [28], *H. rostrata*, native to western South America [28] but a common ornamental throughout the tropics, and *H. wagneriana*, native to Costa Rica and Panama [29]. Furthermore, because so little is known of this unusual plant behavior, we also sought to replicate the results of the original study in the native *H. tortuosa,* an exercise rarely undertaken in experimental ecology [30].

We selected two hummingbird species with different bill morphologies and foraging behaviors as "treatments" in order to accentuate differences in morphological matching to and nectar depletion from the range of flower shapes exemplified by the four *Heliconia* species (Fig 1). Green hermit hummingbirds (*Phaethornis guy*) are common traplining hummingbirds in the region with long ($\bar{x}$ = 41.90 mm, s_x= 1.52 mm), moderately decurved bills ($\bar{k} =$ 0.022 mm^{-1}, s_k = 0.004 mm^{-1}, n = 27 birds of mixed sex, where k_i is the curvature of the ith bill measured as the inverse of the radius of the arc of the bill – see Temeles et al. [15]). Rufous-tailed hummingbirds (*Amazilia tzacatl*) are common territorial hummingbirds with short ($\bar{x}$ = 21.60 mm, s_x = 1.55 mm, n = 14 birds of mixed sex), slightly decurved bills ($\bar{k} =$ 0.016 mm^{-1}, s_k = 0.002 mm^{-1}; Fig 1).

Fig 1. Morphological mismatch between the hummingbird and *Heliconia* species used in experiments. The average mismatch ($\hat{\Delta}$) between a plant and hummingbird species was measured as the Euclidean distance between a flower and a bird's bill in the $\mathbb{R}^2$ trait space, where one axis was the total length of a bill or flower (mm) and the other was the radius of the arc along the outside edge of the flower or bill (mm). We then computed the mean ($\hat{\Delta}$) and standard deviation

141 (s) of the distances between each bill-flower pair, where n is the number of pairwise
142 comparisons.
143
144 Assuming that morphologically matched floral visitors increase the numbers of
145 successful pollen tubes for all *Heliconia* species, we predicted the following: 1) For *H.*
146 *wagneriana* and *H. tortuosa*, we would predict greater numbers of pollen tubes in flowers visited
147 by green hermit hummingbirds compared to rufous-tailed hummingbirds due to long and curved
148 flowers (Fig 1). 2) Because both *H. hirsuta* and *H. rostrata* have shorter, straighter flowers and
149 both hummingbird bill shapes approximate the shape of the flowers well (Fig 1), we would not
150 predict a large difference in the number of pollen tubes between flowers visited by green hermits
151 and those visited by rufous-tailed hummingbirds. We therefore used hand pollinations as a
152 control treatment in all experiments since hand pollinations do not replicate the physical
153 characteristics of a visit by a morphologically matched pollinator aside from pollen deposition.
154 Hence, we would predict the fewest pollen tubes in flowers pollinated by hand for all plant
155 species. Furthermore, this helped us control for potentially low genetic diversity in the pollen
156 pool since the control flowers (hand pollination only) and the treatment flowers (hand pollination
157 followed by a visit from a pollen-free hummingbird) both received pollen by hand from the same
158 donor.
159

160 Aviary experiments

161 To test whether pollen germination and tube growth is dependent on interactions with
162 morphologically matched floral visitors, we conducted 110 single-visit experiments (n = 214
163 flowers from 54 plants; see Table 1 for the number of replicates per treatment) with captive

hummingbirds inside portable aviaries. The aviaries measured 2 meters tall and one meter on a side and could be quickly assembled around live plants (S1 File). In these experiments, we used only virgin flowers that had been covered with mesh bags prior to anthesis in order to preclude pollination by free-ranging pollinators. Flowers were not emasculated, however, due to extremely low numbers of pollen tubes in emasculated flowers in natural settings (A. S. Hadley, *unpublished data*).

Table 1: Sample sizes for individual plants (grouping factor) and flowers (experimental units) for each species × treatment combination.

	Hand pollination (HP)*	**Hand pollination + short-billed bird (SB)**	**Hand pollination + long-billed bird (LB)**	**Hand pollination (HP)****	**Hand pollination + pipette**	**Hand pollination + nectar extraction**	**Nectar extraction + hand pollination**
H. hirsuta							
Plants	7	4	5	0	0	0	0
Flowers	11	5	7	0	0	0	0
H. rostrata							
Plants	19	13	10	13	7	15	5
Flowers	39	25	19	24	8	38	11
H. tortuosa							
Plants	10	8	6	10	7	10	4
Flowers	16	10	11	27	7	36	5
H. wagneriana							
Plants	12	6	11	11	7	8	4
Flowers	31	17	23	17	7	12	10

*Hand pollination controls for aviary experiments
**Hand pollination controls for nectar removal experiments

178 We selected inflorescences based on the availability of two virgin flowers and erected the
179 aviary around the whole plant. We then randomly assigned one of the flowers as a control flower
180 that received hand-pollination but no visit from a bird (HP treatment). The remaining flower was
181 hand-pollinated with pollen from the same donor flower, then allowed a visit by either a pollen-
182 free short-billed hummingbird (SB treatment; n = 14 *A. tzacatl* individuals used in experiments)
183 or a pollen free long-billed hummingbird (LB treatment; n = 12 *P. guy* individuals used in
184 experiments). To ensure the birds were free of pollen before the visit to the focal flower, we
185 cleaned them using a soft paint brush and damp cotton swab under 20x magnification prior to
186 releasing them into the aviary. Thus, flowers were the experimental units and individual plants
187 were treated as a blocking effect to account for potential dependence among measurements on
188 flowers from the same plant. Where possible, plants received all treatments (often on separate
189 days).

190 By hand-pollinating all flowers using pollen from an arbitrarily selected pollen donor, we
191 were able to control for differences in the quality of pollen delivered by the different pollinator
192 species. Indeed, we could not perfectly standardize the quantity of pollen grains at the stigmatic
193 surface because the size of *Heliconia* pollen grains makes it impractical to quantify the number
194 of grains in the field; however, we attempted to reduce variation in the quantity of pollen
195 available to the flowers by having the same experimenter apply pollen in an even layer across the
196 stigmatic surface with a toothpick under 20x magnification for every flower.

197 After the hummingbird visited the treatment flower (evidenced by bill insertion and a
198 clear attempt to feed from the flower), we terminated the experiment and checked the stigma
199 again to ensure that pollen was still present and in an even layer on the stigmatic surface before
200 again covering the flowers with mesh bags. All flowers were collected the following day, the

201 styles removed and preserved in formalin acetyl-acid, and scored for pollen tubes using epi-
202 florescence microscopy [18,23] (see S1 File for more information). All aviary experiments were
203 conducted during the 2018 and 2019 dry seasons (Feb-Mar).
204

205 Tests for a mechanism

206 We conducted additional experiments to test hypotheses of the mechanism of pollinator
207 recognition. Betts et al. [18] found increased pollen tube rates in flowers from which nectar was
208 removed compared to hand pollination alone. As an independent test of whether nectar removal
209 provides a cue to which plants respond, we manually extracted nectar from flowers of three of
210 the four species (*H. hirsuta* did not produce flowers regularly enough to conduct the full suite of
211 experiments) and compared pollen tube rates to control flowers that were hand-pollinated on the
212 same day. Alternatively, it is possible that the long-billed hummingbirds trigger a mechanical
213 cue [31] when they insert their bills into the flower. To test whether we could induce an increase
214 in pollen tube success rates using a mechanical stimulus, we molded a pipette tip to match the
215 curvature of the focal flower. We then inserted the pipette tip as a hummingbird would insert its
216 bill but did not remove any nectar. Finally, because we were unable to perfectly replicate the
217 timing of events in a natural pollinator visit in which nectar removal and pollen deposition
218 happen concurrently, we conducted some experiments in which we hand pollinated before
219 manually removing nectar and some in which we hand pollinated after removing nectar.
220 Differences in these pollen tube rates may indicate the importance of the timing of pollen
221 transfer and nectar removal or bill insertion (see S1 File for more detail).

222 Statistical methods

We separately analyzed pollen tube count data from each set of experiments (i.e., aviary experiments as one dataset and nectar removal experiments as a second dataset). We fit hierarchical Poisson models of pollen tube counts using a Bayesian regression approach implemented in the R package 'rstan' [32,33]. We included effects for each plant of each species (i.e., random blocking effects) to account for potentially correlated observations that could arise from scoring pollen tubes in multiple flowers from the same plant (since individual plants received more than one treatment). A Bayesian approach allowed us flexibility in defining the variance structure such that plant-level effects are considered exchangeable draws from distributions with a variance unique to each plant species. We assumed plant effects are distributed normally (on the log scale) with mean of zero and unknown, species-specific variance, and used weakly informative Gaussian priors for all regression coefficients (see S1 File for full model specification and prior justification). Furthermore, we defined our priors for regression coefficients to concentrate mass around zero such that evidence of an effect needs to be strong to shift the posterior distribution away from zero; our effect sizes are therefore conservative.

We assessed HMC chain convergence using the $\hat{R}$ statistic, checking that the value was below 1.1 for all parameters being estimated [34] and used posterior predictive checks for a range of summary statistics to evaluate model fits. Below, we report posterior mean pollen tube counts per style for a given treatment and plant species as $\hat{\lambda}$ with a subscript indicating the treatment and plant species. We use *HP*, *SB*, *LB*, *HPNE*, *NEHP,* and *BM* to indicate the treatment. Treatment codes are as follows: *HP* identifies the hand-pollinated control flowers; *SB* indicates the treatment in which we hand pollinated flowers, then allowed a clean, rufous-tailed hummingbird (short, straight bill) to visit; *LB* indicates the treatment in which we hand

pollinated flowers, then allowed a pollen-free green hermit hummingbird (long bill) to visit; *HPNE* identifies the treatment in which we hand pollinated the flowers then manually removed the nectar; *NEHP* identifies the treatment in which we hand pollinated the flowers after removing nectar; and *BM* identifies the treatment in which we inserted a pipette tip but did not attempt to remove nectar. We use the letters *h*, *r*, *t*, and *w* to identify *H. hirstuta*, *H. rostrata*, *H. tortuosa*, and *H. wagneriana* in the subscripts (respectively). We additionally report differences between treatments as the fold change in pollen tube rates and use the notation $\hat{\lambda}_{i,j}/\hat{\lambda}_{k,j}$ for the fold change between treatments i and k for plant species j. Ninety-five percent credible intervals for posterior estimates are presented in square brackets.

Results

When we compared pollen tube counts in hand-pollinated control flowers (HP) and those that were visited by a pollen-free, morphologically matched hummingbird, we found evidence that a visit by a matched hummingbird increases pollen tube rates for *H. tortuosa* over hand pollination alone (Fig 2). Pollen tube rates were 4.98 times greater [1.59, 12.59] following visits from long-billed hummingbirds compared to the control treatments with only hand pollination. However, short-billed hummingbird visits did not boost pollen tube rates for *H. tortuosa* above the hand-pollinated controls ($\hat{\lambda}_{HP,t}$= 0.23 tubes per style [0.05, 0.56]; $\hat{\lambda}_{SB,t}$ = 0.28 tubes per style [0.048, 0.76]; Fig 2). Thus, the pollen tube rate in flowers visited by morphologically matched hummingbirds was greater (4.51 times greater [1.07, 13.88]) than the pollen tube rate in flowers visited by morphologically mismatched hummingbirds (Fig 1).

268 **Fig 2.** Pollen tube rates in flowers that received hand pollination only (HP) and those visited by a
269 pollen-free hummingbird following hand pollination. Flowers were visited by either short-billed
270 (SB), rufous-tailed hummingbirds (*Amazilia tzacatl*) or long-billed (LB) green hermit
271 hummingbirds (*Phaethornis guy*). Thick, interior error bars show 80% posterior credible
272 intervals and thin error bars show 95% credible intervals. The grey, horizontal dashed lines show
273 estimates ($\pm SE$) of the pollen tube rates in flowers left open to free ranging pollinators. Note
274 that the range on the y-axis differs for *H. rostrata*.
275
276 In *H. rostrata* flowers, pollen tube rates in those visited by hummingbirds were greater
277 than hand pollination alone regardless of the bird species used in experiments. The estimated
278 rates were nearly identical in flowers visited by long-billed hummingbirds and those visited by
279 short-billed hummingbirds, but were nearly double the rate in hand pollinated controls ($\hat{\lambda}_{HP,r}$ =
280 0.39 tubes per style [0.23, 0.62]; $\hat{\lambda}_{SB,r}$ = 0.70 tubes per style [0.39, 1.11]; $\hat{\lambda}_{LB,r}$ = 0.72 tubes per
281 style [0.38, 1.22]; Fig 2).
282 For *H. hirsuta* and *H. wagneriana,* single visits from cleaned hummingbirds did not
283 enhance pollen tube success rates above hand pollination alone (Fig 2), and pollen tube rates in
284 *H. wagneriana* flowers that were visited by clean birds were actually reduced. The number of
285 pollen tubes per style in *H. wagneriana* flowers visited by green hermits were a little more than
286 half those of hand pollinations ($\hat{\lambda}_{LB,w}/\hat{\lambda}_{HP,w} =$ 0.64 [0.36, 1.07]) and short-billed hummingbird
287 visits yielded pollen tube rates less than half of hand pollination treatments ($\hat{\lambda}_{SB,w}/\hat{\lambda}_{HP,w} =$ 0.43
288 [0.18, 0.82]; Fig 2).
289 When we experimentally removed nectar using pipette tips to test the hypothesis that
290 differential nectar removal may be the mechanism for pollinator recognition, the effect of nectar

removal on pollen tube rates for *H. rostrata* and *H. tortuosa* was negligible (Fig 3). We did find some evidence that our nectar removal treatments had a positive effect on pollen tube germination relative to hand-pollinations alone for *H. wagneriana* ($\hat{\lambda}_{HP,w}$ = 0.42 [0.09, 1.04]; $\hat{\lambda}_{HPNE,w}$ = 0.89 [0.26, 2.07]; Fig 3), but this effect is driven in part by a small number of influential observations (>5 pollen tubes found in 2 styles), and disappears after removing them, so we caution readers in their interpretation of this result.

Fig 3. Results from experimental tests of the effect of nectar depletion on pollen tube rates. We used hand-pollination (HP) as a control treatment and compared pollen tube rates in flowers that received the control treatment to those in flowers that received out-cross pollen by hand either before (HP + nectar extraction) or after (Nectar extraction + HP) manual removal of the nectar in the flower. As a test of whether pollen germination success could be boosted after the mechanical stimulus of a hummingbird inserting its bill to drink from the flower, we tested for an effect of pipette insertion without removing any nectar (HP + pipette). Interior, thick error bars show 80% posterior credible intervals and narrow bars show 95% credible intervals.

Inserting a hummingbird bill mimic (i.e., pipette tip) into flowers as a mechanical signal without removing nectar also did not induce substantially higher pollen tube rates in any of the tested species (Fig 3), and, in all cases, hand pollinating flowers after removing nectar resulted in the fewest pollen tubes per style out of all treatments, generally about half of hand pollination alone ($\hat{\lambda}_{NEHP,r}/\hat{\lambda}_{HP,r}$ = 0.44 [0.09, 1.24]; $\hat{\lambda}_{NEHP,t}/\hat{\lambda}_{HP,t}$ = 0.64 [0.05, 2.33]; $\hat{\lambda}_{NEHP,w}/\hat{\lambda}_{HP,w}$ = 0.44 [0.10, 1.15]).

Discussion

We tested for generality of pollinator recognition in *Heliconia,* which may act in conjunction with physical exploitation barriers, such as long and/or curved corolla tubes, to filter the pollinator community. If the capacity for plants to actively filter floral visitors based on morphological trait matching is widespread, this would have implications for the robustness of plant-pollinator communities. Given local extinction or reduced densities of morphologically matched pollinators, mismatched pollinators may alter their foraging behaviors to exploit newly available resources [35–38]; however, visits from mismatched pollinators would not compensate for the pollination services lost to a plant with a pollinator recognition mechanism, even if they deposit pollen at the stigma. This could increase the likelihood of coextinctions.

We provide evidence that the pollinator recognition behavior could occur beyond a single species. Experiments that allowed us to standardize pollen quality and minimize variation in the quantity of pollen at the stigmatic surface showed that pollen tube rates in flowers pollinated by hand were elevated if also visited by pollen-free hummingbirds with morphologically matched bills compared to hand-pollination alone in two of four species tested. Furthermore, flowers that were visited by birds with mismatched bill shapes did not enhance pollen tube rates compared to hand pollination alone in *H. tortuosa* flowers. These results substantiate the findings of Betts et al. [18] for *H tortuosa,* and provide new evidence supporting that visits by morphologically matched pollinators may enhance pollen tube success in *H. rostrata*. While the pollen tube rates did not differ among the *H. rostrata* flowers that were visited by green hermit hummingbirds and those visited by rufous-tailed hummingbirds, the relatively low pollen tube counts in the flowers

336 that received hand pollination alone indicate that a poorly matched floral visitor may not enhance
337 pollen tube rates. We discuss this further below.
338

339 The mechanism of pollinator recognition

340 Previous work demonstrated increased pollen tube rates with manual nectar extraction
341 treatments compared to hand-pollinations alone in *H. tortuosa* [18]. Betts et al. [18] hypothesized
342 that, because birds with well-matched bill morphologies can drain the nectar chamber but those
343 with mismatched bills often cannot [17,18], nectar removal could provide a cue to which plants
344 respond to promote successful pollen tube growth. Our results do not corroborate these findings
345 as we did not find a substantial increase in pollen tube rates for *H. tortuosa* or *H. rostrata*
346 following manual nectar removal treatments. While we found increased pollen tube rates in *H.*
347 *wagneriana* flowers following manual nectar removal (Fig 3), this result is tenuous because the
348 pollen tube counts in the flowers from which we removed nectar are similar to the pollen tube
349 counts in our hand pollinated flowers from the aviary experiments (Figs 2 and 3).
350 An alternative explanation for the increase in pollen tube rates following bird visits (*H.*
351 *tortuosa* and *H. rostrata*; Fig 2) is that pollen supplied by hand was subsequently removed or
352 rearranged by pollen-free hummingbirds. This could reduce pollen competition at the stigmatic
353 surface, potentially promoting pollen tube germination [39]. However, there are two primary
354 reasons we find this explanation unlikely. First, both long-billed and short-billed hummingbirds
355 contact the stigmas of *H. tortuosa*, but only the long-billed hummingbird visits resulted in
356 increased pollen tube counts. Second, neither the pollen tube rates in flowers that received the
357 bill mimic treatment nor the flowers for which pollen was applied before nectar removal
358 (potential for pollen rearrangement) differed strongly from those in the hand pollinated control

flowers, which have no potential for pollen rearrangement (Fig 3). We therefore do not believe that a parsimonious explanation is one in which morphologically matched hummingbirds arrange pollen at the stigmatic surface in precisely the right way to stimulate germination. We note, however, that if true, this could still provide a cryptic pollinator recognition mechanism. In summary, the evidence that nectar removal provides the cue for pollinator recognition is equivocal and further experiments are necessary to verify nectar removal or establish a new mechanism.

Implications of pollinator recognition

Heliconia tortuosa and *H. rostrata* represent two distant branches of the >200-branch Heliconiaceae phylogeny [22]. Given the positions of these two taxa on the tree, the mechanism that allows pollinator recognition in *H. tortuosa* could be basal and potentially prevalent in the Heliconiaceae and other related families. While, for *H. rostrata*, the mechanism did not appear to filter either bird species used here, the response to bird visitation compared to hand pollination alone suggests the potential for a pollinator filter. The realized degree of specialization in *Heliconia*-hummingbird pollination systems may therefore be more specialized than what interaction data (flower visits) show. Data on pollination efficiencies of morphologically matched and mismatched pollinators could help to support or refute this idea, but little is known about pollination of these *Heliconia* species within their native geographic ranges. More generally, mismatched pollinators are often less efficient pollinators [17,40–43], but this is largely thought to be due to differences in pollen transfer efficiency and not active filtering by the plant. More data are needed on these and other taxa to understand the implications of pollinator recognition in natural populations.

382 In forest fragments around Coto Brus, mismatched hummingbirds account for c.a. 10% of
383 honest visits (those in which the visitor contacts the reproductive organs of the flower) to *H.*
384 *tortuosa* (K. Leimburger, *unpublished data*), and this proportion likely increases in isolated
385 fragments where morphologically matched hummingbirds are less common [44–46]. Indeed, *H.*
386 *tortuosa* plants in fragmented forests show reduced seed sets compared to those in continuous
387 forest [47], presumably due to a paucity of morphologically matched hummingbirds. Pollinator
388 recognition is therefore likely to be an additional pollinator filter acting in conjunction with
389 morphological barriers that often result in only imperfect resource partitioning by floral visitors
390 [16,48–50].

391 From an evolutionary standpoint, we agree with Betts et al. [18] that filtering the short-
392 billed, territorial hummingbirds could promote outcrossing and enhance the genetic diversity of
393 pollen grains that reach the ovules. The hummingbirds of Coto Brus with bill morphologies that
394 complement the flowers of *H. tortuosa* carry taxonomically diverse pollen loads, but the ratio of
395 *Heliconia* to other pollen tends to be greater than it is on mismatched pollinators [18]. Because
396 these morphologically matched pollinators forage over a larger range [51] and carry high
397 *Heliconia* pollen loads, the pool of conspecific pollen available to a plant on a given visit may be
398 more genetically diverse than on a visit from a mismatched pollinator. This would be adaptive if
399 plants suffer from inbreeding depression. Indeed, Torres-Vanegas et al. [21,52] found genetic
400 signatures in *H. tortuosa* populations that are consistent with this hypothesis; inbreeding was
401 greatly reduced in large, connected forest patches where morphologically matched, traplining
402 hummingbirds were more prevalent.

403 In *H. rostrata* styles, visits from clean hummingbirds to hand-pollinated flowers also
404 increased pollen tube rates, but regardless of the bird species. The effects in *H. rostrata* were

405 nearly identical for two species of hummingbirds with notably different morphologies (Fig 1).
406 However, given the relatively short, straight corolla of *H. rostrata* (Fig 1), both hummingbird
407 species we used for experiments were able to achieve high morphological matches and might not
408 be expected to differ in their visitation characteristics, such as nectar consumption. We did not
409 destructively sample flowers after hummingbird visits to measure the nectar remaining, but both
410 species of birds can be seen drinking from *H. rostrata* flowers in recorded videos (S1 and S2
411 videos).

412 In Peru, seven hummingbird species of various sizes and with various bill shapes have
413 been observed visiting *H. rostrata*, but nothing is known of their pollination efficiencies [53].
414 Based on our results showing increased pollen tube rates in bird visited flowers compared to
415 hand pollination, we posit that *H. rostrata* could filter visits from animals without
416 complementary morphologies. This idea is supported by data from Janeček et al. [26] who
417 recorded olive sunbirds (*Cyanomitra olivacea*) and Camaroon sunbirds (*Cyanomitra oritis*)
418 visiting *H. rostrata* flowers in South Africa where it has been introduced. These authors found
419 that *H. rostrata* flowers left open to visits from sunbirds had extremely low pollen tube rates, as
420 did hand-pollinated flowers. While these results are suggestive of a cryptic pollinator filter, it
421 could also be that promoting pollen tube growth after receiving a cue from a pollinator
422 (regardless of the pollinator's identity) could reduce rates of self-pollination, similarly promoting
423 outcrossing. Subsequent floral evolution toward longer and more curved flowers could have
424 resulted in an active and cryptic pollinator filter for *H. tortuosa*, precluding pollen delivered by
425 mismatched pollinators.

426 Conversely, we were not able to detect an effect of hummingbird visits to *H. hirsuta* and
427 even found reduced pollen tube rates in *H. wagneriana* flowers that were visited by

428 hummingbirds relative to hand-pollinations alone. While the mechanisms underlying this result
429 in *H. wagneriana* remain unclear, we identified one way in which this species differs from the
430 others that could produce this result. Gannon et al. [27] discovered that *H. wagneriana* plants
431 have a mechanism for keeping the anthers protected within the perianth and then rapidly
432 extending them as a hummingbird visits. This is thought to protect pollen from desiccation
433 and/or increase pollen transfer to pollinators during the first visit. Once exposed, however, pollen
434 grains desiccate relatively quickly, and often fail to adhere to the stigmatic surface. This may
435 make the pollen grains of *H. wagneriana* especially easy to dislodge. While we checked that
436 pollen was still present on the stigma after a bird visited, the size of *Heliconia* pollen makes
437 exact quantification in the field infeasible. Thus, it is possible that reduced pollen loads after the
438 birds visited resulted in reduced pollen tube counts relative to hand pollination alone.
439 Using camera traps, Gannon et al. [27] found that c.a. 97% of the visits to open *H.*
440 *wagneriana* flowers around Las Cruces were by traplining species with morphologically
441 matched bill shapes. Similarly, Snow and Snow [54] report only green hermit and rufous-
442 breasted hermit (*Glaucis hirsutus*) visitors at *H. hirsuta* flowers in Trinidad (part of its native
443 range), both of which have well-matched bills. While we did not find evidence for pollinator
444 recognition in these two *Heliconia* species, it is possible that a pollinator recognition mechanism
445 would not help to filter floral visitors given that morphologically mismatched pollinators may
446 not account for a considerable proportion of visits.
447

448 # Conclusions

449 Our results help to highlight the potential for cryptic plant behaviors that could result in
450 pollinator filters and therefore cryptic specialization. Detailed and manipulative experiments to

assess realized pollination network structure and vulnerability to disturbance are therefore warranted [4–7]. A starting point to identify possible cryptic pollinator filters is the comparison of pollen tube rates (or other proxies/metrics for reproduction) in hand-pollinated flowers versus open pollination. Reduced pollen tube success in hand pollinated flowers compared to open pollination would identify the potential for more complex dynamics than the simple placement of pollen on the stigma. Notably, Pedersen and Kress [24] report a c.a. four-fold increase in pollen tube rates in *Heliconia paka* flowers that were visited by honeyeaters compared to those pollinated by hand. These results would be consistent with what we would predict for *H. paka* given a pollinator recognition mechanism. More generally, Young and Young [39] report that hand-pollinated flowers had reduced reproductive output compared to open-pollinated flowers for 17 of 52 plant species from highly divergent lineages. We know of no follow-up experiments with these or related taxa, but we urge others to conduct similar experiments to those presented here to examine the potential for cryptic specialization in other pollination systems.

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Data accessibility

Data from pollination experiments and all R code necessary to reproduce the results can be found on a public Github repository (https://github.com/Dusty-Gannon/PR-in-Heliconia).

Author contributions

MGB, ASH, and DGG designed experiments. DGG analyzed the data and wrote the original version of the manuscript. All authors contributed to data collection and critical review of the manuscript.

Ethical statement

All experimental methods involving hummingbirds were approved by the Oregon State University Animal Care and Use Committee (Animal Care and Use Permit 5020) and all international research guidelines and practices were followed.

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Supporting information

S1 video. Green hermit hummingbird (*Phaethornis guy*) visiting and drinking nectar from a *Heliconia rostrata* flower.

S2 video. Rufous-tailed hummingbird (*Amazilia tzacatl*) visiting and drinking nectar from a *Heliconia rostrata* flower.

S1 File. Supplementary methods.

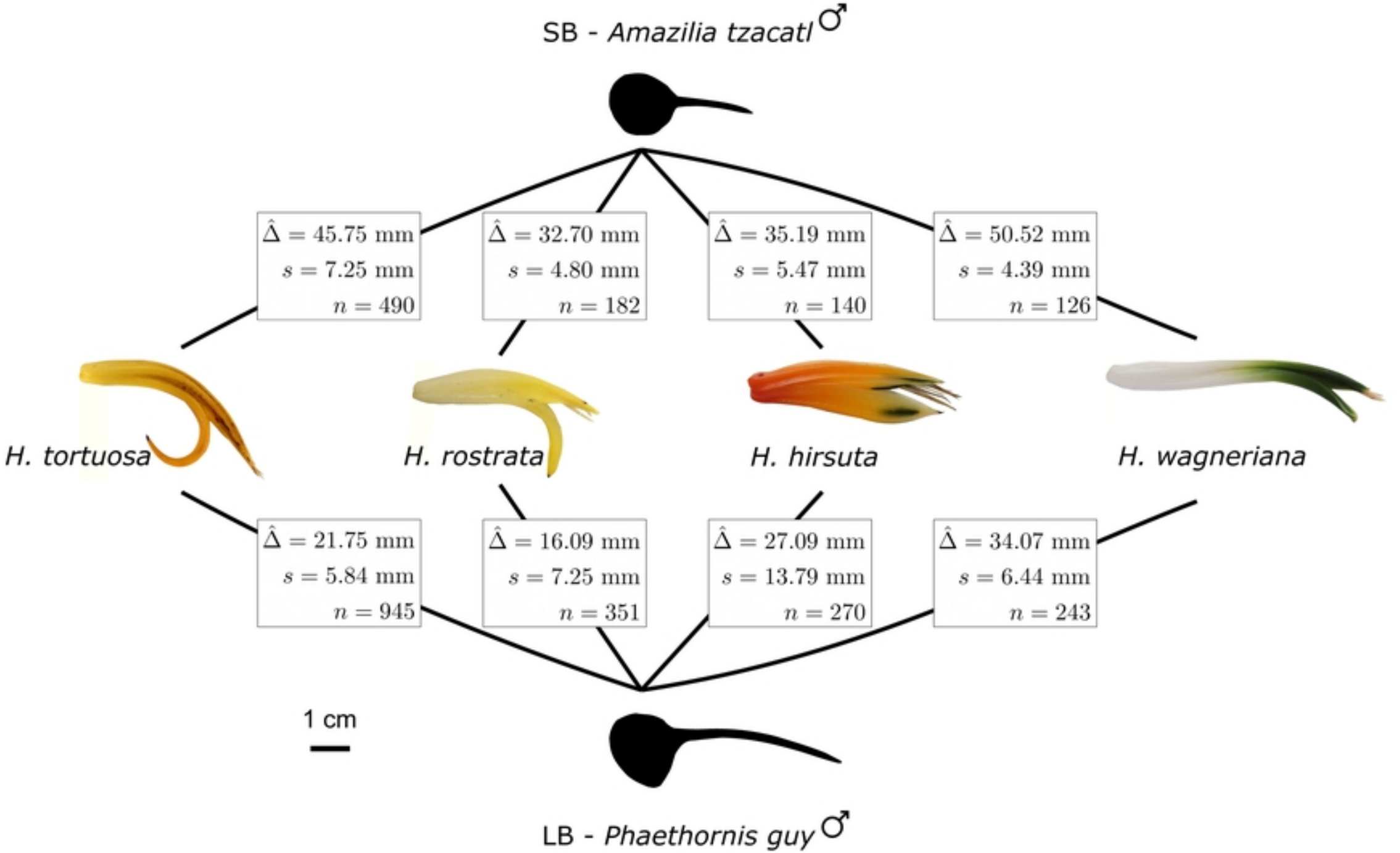

Figure 1. Mismatch

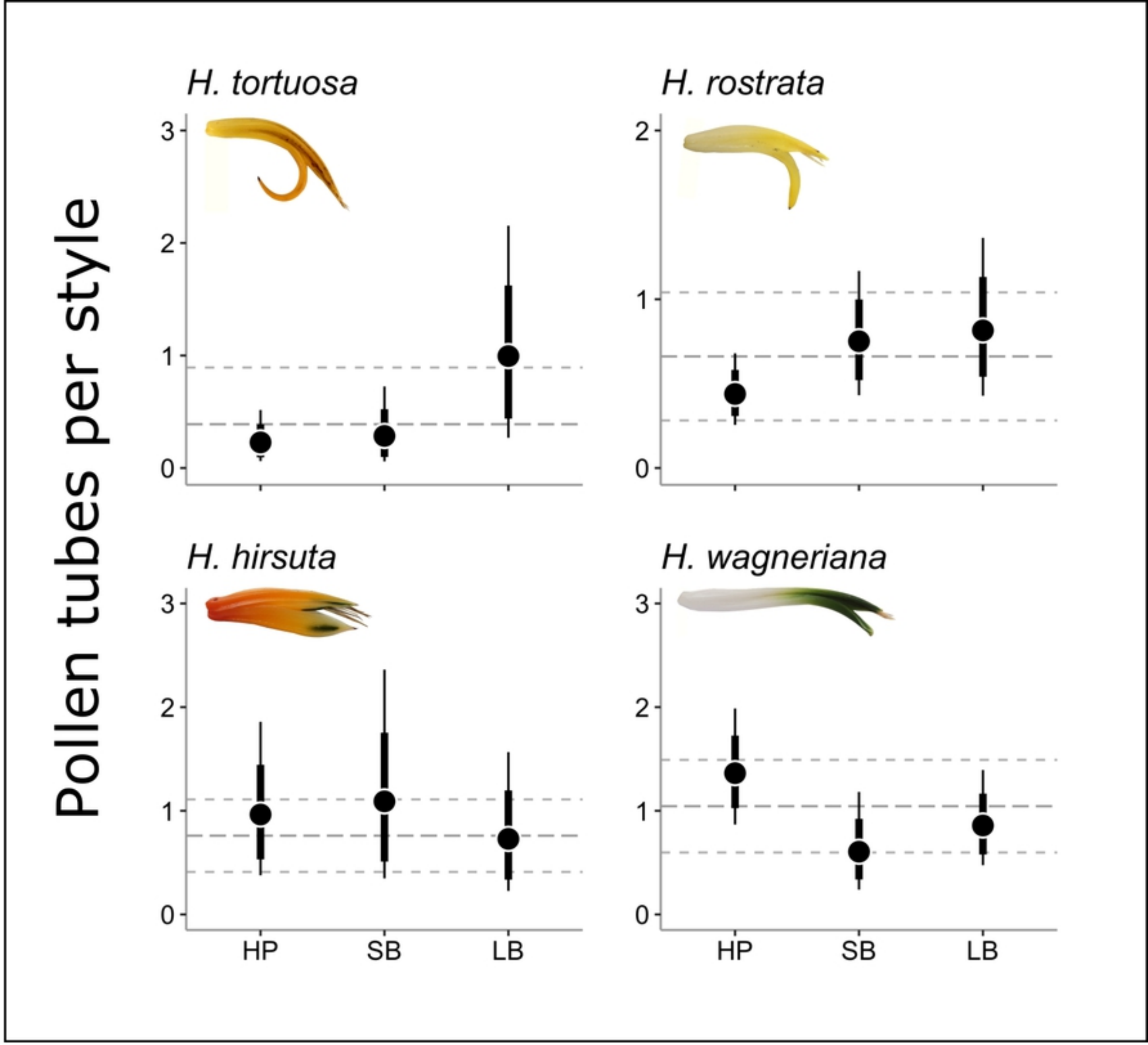

Figure 2. Results from aviary experiments

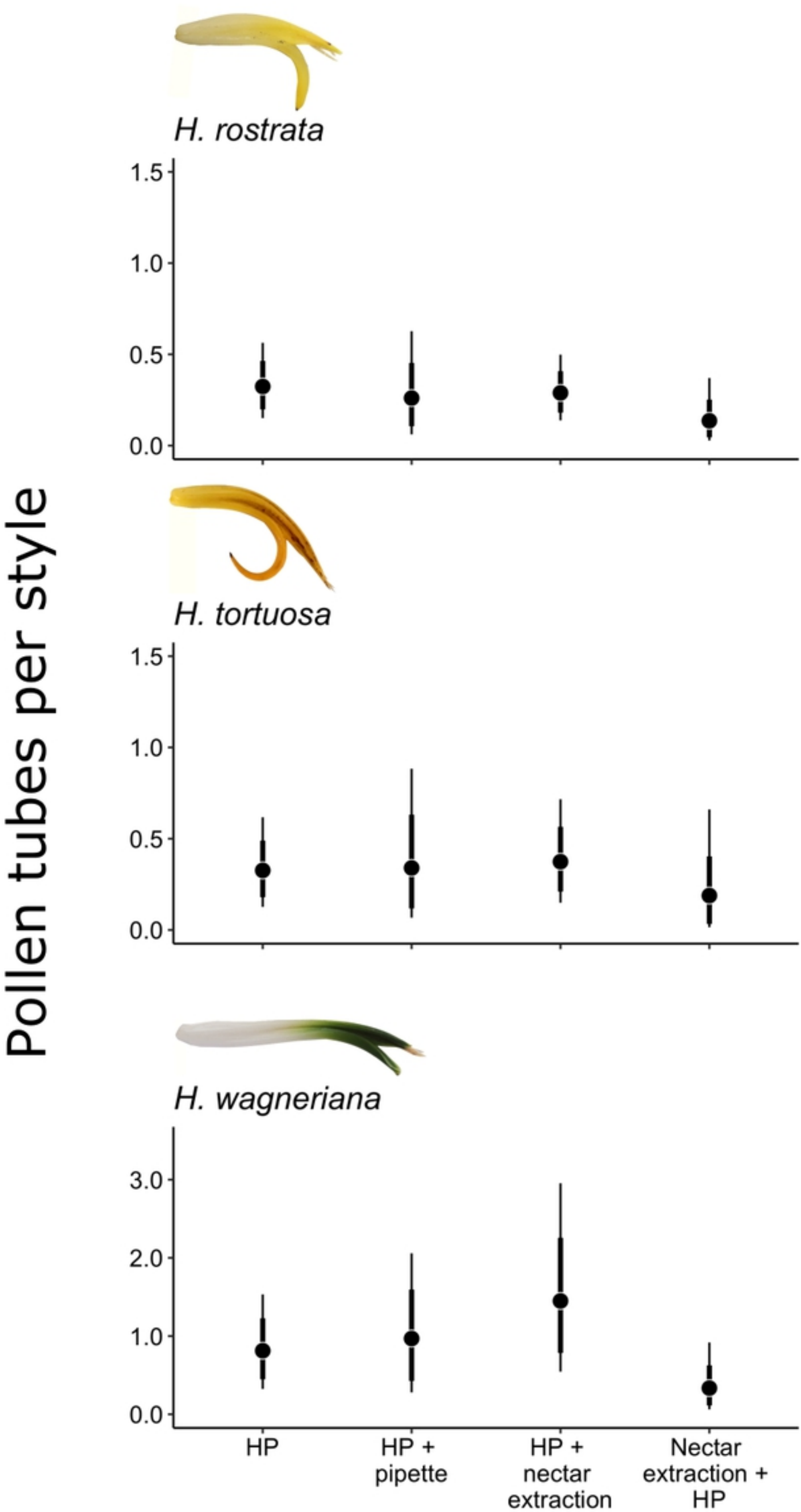

Figure 3. Results from nectar removal experime