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 ( = 41.90 mm, = 1.52 mm), moderately decurved bills ( 0.022 , = 0.004 , *n* = 27 birds of mixed sex, where *ki* is the curvature of the *i*th 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 ( = 21.60 mm, = 1.55 mm, *n* = 14 birds of mixed sex), slightly decurved bills ( 0.016 , = 0.002 ; Fig 1).

**Fig 1.** Morphological mismatch between the hummingbird and *Heliconia* species used in experiments. The average mismatch () between a plant and hummingbird species was measured as the Euclidean distance between a flower and a bird’s bill in the 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 () and standard deviation (*s*) of the distances between each bill-flower pair, where *n* is the number of pairwise comparisons.

Assuming that morphologically matched floral visitors increase the numbers of successful pollen tubes for all *Heliconia* species, we predicted the following: 1) For *H. wagneriana* and *H. tortuosa*, we would predict greater numbers of pollen tubes in flowers visited by green hermit hummingbirds compared to rufous-tailed hummingbirds due to long and curved flowers (Fig 1). 2) Because both *H. hirsuta* and *H. rostrata* have shorter, straighter flowers and both hummingbird bill shapes approximate the shape of the flowers well (Fig 1), we would not predict a large difference in the number of pollen tubes between flowers visited by green hermits and those visited by rufous-tailed hummingbirds. We therefore used hand pollinations as a control treatment in all experiments since hand pollinations do not replicate the physical characteristics of a visit by a morphologically matched pollinator aside from pollen deposition. Hence, we would predict the fewest pollen tubes in flowers pollinated by hand for all plant species. Furthermore, this helped us control for potentially low genetic diversity in the pollen pool since the control flowers (hand pollination only) and the treatment flowers (hand pollination followed by a visit from a pollen-free hummingbird) both received pollen by hand from the same donor.

**Aviary experiments**

To test whether pollen germination and tube growth is dependent on interactions with morphologically matched floral visitors, we conducted 110 single-visit experiments (*n* = 214 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

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

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

After the hummingbird visited the treatment flower (evidenced by bill insertion and a clear attempt to feed from the flower), we terminated the experiment and checked the stigma again to ensure that pollen was still present and in an even layer on the stigmatic surface before again covering the flowers with mesh bags. All flowers were collected the following day, the styles removed and preserved in formalin acetyl-acid, and scored for pollen tubes using epi-florescence microscopy [18,23] (see S1 File for more information). All aviary experiments were conducted during the 2018 and 2019 dry seasons (Feb-Mar).

**Tests for a mechanism**

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

**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.

Below, we report mean pollen tube counts per style estimated from the model fit for a given treatment and plant species as 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 for the fold change between treatments and for plant species . This is a natural way in which to report differences between treatments given the log link function. Ninety-five percent confidence intervals for estimates are denoted with followed by an interval estimated from 1,000 bootstrapped mean predictions [1].

**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 increased pollen tube rates for *H. tortuosa* over hand pollination alone (Fig 2)*.* Pollen tube rates were 6.91 times greater following visits from long-billed hummingbirds compared to the control treatments with only hand pollination (; tubes per style, ; tubes per style, ). However, short-billed hummingbird visits did not boost pollen tube rates for *H. tortuosa* above the hand-pollinated controls( tubes per style, ; Fig 2). Thus, the pollen tube rate in flowers visited by morphologically matched hummingbirds was greater (3.32 times greater, ) than the pollen tube rate in flowers visited by morphologically mismatched hummingbirds (Figs 1&2).

**Fig 2.** Pollen tube rates in flowers that received hand pollination only (HP) and those visited by a pollen-free hummingbird following hand pollination. Flowers were visited by either short-billed (SB) rufous-tailed hummingbirds (*Amazilia tzacatl*) or long-billed (LB) green hermit hummingbirds (*Phaethornis guy*). Error bars show the estimate plus or minus bootstrapped standard errors. The grey, horizontal dashed lines show estimates () of the pollen tube rates in flowers left open to free ranging pollinators.

In *H. rostrata* flowers, pollen tube rates in those visited by hummingbirds were greater than hand pollination alone regardless of the bird species used in experiments. The estimated rates were similar in flowers visited by long-billed hummingbirds and those visited by short-billed hummingbirds, but were approximately double the rate in hand pollinated controls ( tubes per style, ; tubes per style, ; tubes per style, ; Fig 2).

For *H. hirsuta* and *H. wagneriana,* single visits from cleaned hummingbirds did not enhance pollen tube success rates above hand pollination alone(Fig 2), and pollen tube rates in *H. wagneriana* flowers that were visited by clean birds were actually reduced. The number of pollen tubes per style in *H. wagneriana* flowers visited by green hermits were a little more than half those of hand pollinations and short-billed hummingbird visits yielded pollen tube rates less than half of hand pollination treatments ( tubes per style, ; tubes per style, ; tubes per style, ; Fig 2).

When we experimentally removed nectar using pipette tips to test the hypothesis that 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* ( = 0.42 [0.09, 1.04]; = 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). Error bars show estimates of the mean plus or minus bootstrapped standard errors.

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 ( = 0.44 [0.09, 1.24]; = 0.64 [0.05, 2.33]; = 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 that received hand pollination alone indicate that a poorly matched floral visitor may not enhance pollen tube rates. We discuss this further below.

**The mechanism of pollinator recognition**

Previous work demonstrated increased pollen tube rates with manual nectar extraction treatments compared to hand-pollinations alone in *H. tortuosa* [18]. Betts et al. [18] hypothesized that, because birds with well-matched bill morphologies can drain the nectar chamber but those with mismatched bills often cannot [17,18], nectar removal could provide a cue to which plants respond to promote successful pollen tube growth. Our results do not corroborate these findings as we did not find a substantial increase in pollen tube rates for *H. tortuosa* or *H. rostrata* following manual nectar removal treatments. While we found increased pollen tube rates in *H. wagneriana* flowers following manual nectar removal (Fig 3), this result is tenuous because the pollen tube counts in the flowers from which we removed nectar are similar to the pollen tube counts in our hand pollinated flowers from the aviary experiments (Figs 2 and 3).

An alternative explanation for the increase in pollen tube rates following bird visits (*H. tortuosa* and *H. rostrata*; Fig 2) is that pollen supplied by hand was subsequently removed or rearranged by pollen-free hummingbirds. This could reduce pollen competition at the stigmatic surface, potentially promoting pollen tube germination [39]. However, there are two primary reasons we find this explanation unlikely. First, both long-billed and short-billed hummingbirds contact the stigmas of *H. tortuosa*, but only the long-billed hummingbird visits resulted in increased pollen tube counts. Second, neither the pollen tube rates in flowers that received the bill mimic treatment nor the flowers for which pollen was applied before nectar removal (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.

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

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

In *H. rostrata* styles, visits from clean hummingbirds to hand-pollinated flowers also increased pollen tube rates, but regardless of the bird species. The effects in *H. rostrata* were nearly identical for two species of hummingbirds with notably different morphologies (Fig 1). However, given the relatively short, straight corolla of *H. rostrata* (Fig 1),both hummingbird species we used for experiments were able to achieve high morphological matches and might not be expected to differ in their visitation characteristics, such as nectar consumption. We did not destructively sample flowers after hummingbird visits to measure the nectar remaining, but both species of birds can be seen drinking from *H. rostrata* flowers in recorded videos (S1 and S2 videos).

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

Conversely, we were not able to detect an effect of hummingbird visits to *H. hirsuta* and even found reduced pollen tube rates in *H. wagneriana* flowers that were visited by hummingbirds relative to hand-pollinations alone. While the mechanisms underlying this result in *H. wagneriana* remain unclear, we identified one way in which this species differs from the others that could produce this result. Gannon et al. [27] discovered that *H. wagneriana* plants have a mechanism for keeping the anthers protected within the perianth and then rapidly extending them as a hummingbird visits. This is thought to protect pollen from desiccation and/or increase pollen transfer to pollinators during the first visit. Once exposed, however, pollen grains desiccate relatively quickly, and often fail to adhere to the stigmatic surface. This may make the pollen grains of *H. wagneriana* especially easy to dislodge. While we checked that pollen was still present on the stigma after a bird visited, the size of *Heliconia* pollen makes exact quantification in the field infeasible. Thus, it is possible that reduced pollen loads after the birds visited resulted in reduced pollen tube counts relative to hand pollination alone.

Using camera traps, Gannon et al. [27] found that c.a. 97% of the visits to open *H. wagneriana* flowers around Las Cruces were by traplining species with morphologically matched bill shapes. Similarly, Snow and Snow [54] report only green hermit and rufous-breasted hermit (*Glaucis hirsutus*) visitors at *H. hirsuta* flowers in Trinidad (part of its native range), both of which have well-matched bills. While we did not find evidence for pollinator recognition in these two *Heliconia* species, it is possible that a pollinator recognition mechanism would not help to filter floral visitors given that morphologically mismatched pollinators may not account for a considerable proportion of visits.

**Conclusions**

Our results help to highlight the potential for cryptic plant behaviors that could result in 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.

**References**

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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.**