Response to reviewer comments: Testing for generality of pollinator recognition in *Heliconia*

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## \*We wish to be identified to the reviewers.

## We thank Dr. Jennifer Lau, the editor, and the two anonymous reviewers for their thorough and constructive comments on the previous version of this manuscript entitled “Testing for generality of pollinator recognition in *Heliconia*”. Below, we detail our responses to the comments and suggested edits, and the revised manuscript includes grey text for edits specific to the editor’s comments, blue text for edits specific to reviewer one, and orange text for edits specific to the comments of reviewer two. We hope the reviewers find our manuscript much improved.

## Editor comments

**General comments**

First, I very much liked the premise of your study--testing for generality of previous very intriguing findings. I also appreciated the search for a mechanism underlying a potentially very widespread pattern…

**Response**

We greatly appreciate these kind words of encouragement.

#### E.1

* ☒ Addressed in lines 317-334.

First, like Reviewer 2, I wondered if there is any literature on pollinator efficiencies of visitors to these taxa? Or even more general information about whether pollinators with mismatched bill shapes are typically less efficient pollinators in the wild. I would imagine so, but for someone outside the system, I think this needs to be emphasized. It's possible this was investigated for at least H. tortuosa in the Betts et al. 2015 paper, but it was unclear to me from the very brief description provided.

**Response**

This is an excellent question. While there exists some literature on pollination efficiency and trait matching for other taxa, for example the *Heliconia*-hummingbird systems of the West Indies (Temeles et al. 2019), bumble bees and *Solanum rostratum* in Mexico (Solís‐Montero and Vallejo‐Marín 2017), and plant-pollinator networks in the deserts of Argentina (Peralta et al. 2020), knowledge of the pollination efficiencies of the visitors to these *Heliconia* species is very limited. We have included some discussion and relevant sources in the revised Discussion section. Please refer to lines 317-334 for the specific revisions.

### E.2

Like Reviewer 1, I also wondered who the major visitors of these taxa are in the wild (long-billed or short-billed). Some of these details are mentioned briefly in the discussion, but because I think these details are important to evaluating the robustness of your test of the pollinator recognition hypothesis, I think it should be presented earlier in the manuscript and also in more detail. I harp on these natural history questions because I think the answers to them (if available from previous literature or your own studies) could either strengthen or dramatically weaken your conclusions. For example, while I appreciate the discussion of caveats in the discussion, I also worried that by not using the most abundant potential pollinator (at least for one of the taxa, but possibly more?) the comparisons may be difficult to interpret. In other words, if the study is about pollinator recognition but the dominant visitors are not included, then how does that change our interpretation of these findings?

* ☒ Addressed in lines 325-334, 354-362, 376-383, and 384-386.

**Response**

We recognize that if these *Heliconia* species are visited primarily by morphologically matched pollinators, this would refute our hypothesis of pollinator recognition as a pollinator filter. Little is known about the pollinator assemblages for these taxa, but based on data collected by several co-authors on the current paper and some previous research by others, *H. tortuosa* plants receive a considerable number of visits from mismatched pollinators in Coto Brus, Costa Rica, supporting our hypothesis (Morrison and Mendenhall 2020). Furthermore, we elaborate on our arguments that cryptic pollinator filters could increase the likelihood of coextinction events. Given the local extinction of a well-matched pollinator, mismatched pollinators may shift their behaviors to exploit newly available resources. However, if pollen tubes fail to grow following visits from mismatched visitors, a shift in behavior would not compensate the loss of the well-matched pollinator, making coextinction of the plant more likely. We have detailed these arguments more carefully in the revised manuscript.

While we agree that conducting these experiments outside the native ranges of these taxa is not ideal, we now include text on why this makes our methods conservative with respect to identifying species with pollinator recognition (lines 384-386). Specifically, we would expect the differences between hand pollination and visits by cleaned birds from the native ranges to be even greater than what we observed given a pollinator recognition mechanism.

## Reviewer 1 comments

**General comments**

This is a well-written study that describes a series of experiments of general interest to readers of the American Naturalist …

**Response**

We would like to thank the reviewer for the kind comments regarding the writing.

### 1.1

The finding that hand pollinations resulted in fewer pollen tubes than naturally outcrossed flowers exposed to hummingbird visits is not necessarily evidence of pollinator recognition. Rather, it indicates that something about hand pollination does not duplicate the natural pollination process, which beyond the hummingbird itself, could depend upon the timing of the pollinator visit in relation to the life of the flower, the number of hummingbird visits, or nectar removal.

* ☒ Addressed by removal of text from the previous version of the manuscript.

**Response**

We completely agree with this comment and this is why we conducted elaborate hummingbird aviary experiments with known pollen sources and hand pollinations. In the previous version of the manuscript, we included text on the limitations of comparing hand pollination to open pollination (lines 117-125 of the previous version). Nevertheless, we chose to remove those data from this version of the manuscript. This should help shift the focus to the experiments in which we standardized pollen quality between flowers visited by hummingbirds and those used as hand pollinated controls. In these experiments, we were also able to standardize the number of bird visits (limited to 1) and reduce variation in pollen quantity on each style. We do, however, include estimates of the mean pollen tube counts in flowers left open to free-ranging pollinators as a reference in Figure 1.

### 1.2

The conflicting results of this study with Betts et al. 2015 leave the mechanism for pollinator recognition unclear, which the authors note on line 325. It's hard to imagine that birds are rearranging pollen after hand pollination, and I agree with the authors' conclusion that this mechanism seems unlikely (lines 319-321). Nectar removal seems the most likely explanation, and was the explanation offered by Betts et al. 2015. Yet in the current study, hand pollination plus nectar removing using a pipettor did not enhance pollen tube growth, thereby mimicking the results for natural hummingbird pollinators. Possibly, bills or tongues be doing something mechanical inside the flower, such as pushing and relocating the staminode, or triggering something physiological in the nectar chamber. Alternatively, methods of this study may have differed from those in Betts et al., yet it is unclear how or why they would, given that removing nectar with a pipettor and hand-pollinating flowers are pretty straight forward methods. The inconsistent results call into question pollinator recognition and raise issues about methods and/or the genetics of the plants used in the experiments.

* ☒ Addressed in lines 103-106 and 284-311.

**Response**

Indeed, these results are quite puzzling to us as well, and we agree that hummingbird tongues are complex organs with many characteristics we simply cannot reproduce. It remains a possiblility, as suggested, that hummingbird tongues could trigger the response. **However, we argue that the inconsistent results only call into question the *mechanism* of pollinator recognition** **– not the pollinator recognition behavior itself.** We used the same collection of plants in both sets of experiments (the aviary experiments and the nectar removal experiments) and found increased pollen tube rates following bird visits (with hand pollination). If the genetics of these plants was the reason for a lack of a response to nectar removal, we would not expect increased pollen tube rates following visits by clean hummingbirds either.

### 1.3

To what extent might the low number of pollen tubes result from using experimental plants derived from the same genetic stock? Which species were ornamentals, which species were naturalized, and which species were native? Garden or naturalized plants might have low genetic diversity or may have been selected through the breeding process for low reproductive output. Did you check these plants to see whether they were in fact setting fruits and seeds?

* ☒ Confirmed that we checked for seeds in lines 111-113.
* ☒ Acknowledged issue of genetic diversity in lines 143-146 and 384-386.
* ☒ Which are native and which are not? Addressed in lines 117-122.

**Response**

While we agree that the experimental design would be strengthened by conducting these experiments in natural settings using larger populations of each plant species, we were unable to do so here. This is certainly a goal for the future.

In our selection process, we targetted plant species in the living collection at Las Cruces Biological station that were also found on neighboring properties to increase our chances of utilizing a more diverse genetic stock. We additionally checked that each species was setting fruits and seeds, confirming that compatible pollen was available in the area and that it was likely that birds were visiting the flowers despite some being non-native. We include more details on this important point in the revised Methods section (lines 99-146) and in a revised appendix.

### 1.4

How many plants and flowers of each Heliconia species were used in the aviary experiments? In addition, how many birds of each bill morphology were used in these experiments? More precisely, readers need to know the number of experimental units for each Heliconia species x experimental trial treatment.

* ☒ Sample sizes of unique plants and flowers (experimental unit) added as Table 1.
* ☒ Number of hummingbirds of each species added in lines 153-155.

**Response**

We thank reviewer 1 for this request, and added a table with the number of unique plants (blocking effect) and numbers of flowers (experimental units) scored for pollen tubes in each species treatment combination.

### 1.5

How were the aviaries constructed? Were you actually building them around live inflorescences of natural plants? Can you provide a photograph in an appendix?

* ☒ Addressed in lines 159-160 of the main text and 28-30 of Appendix S1.
* ☒ Photograph added to Appendix S1

**Response**

We constructed portable aviaries using a PVC frame and bamboo legs. We sewed a mesh net sleeve that slid easily over the frame. This allowed us to erect aviaries around live inflorescences. We included more details on aviary construction in the revised methods section and in the revised appendix and included a photograph of the aviaries.

### 1.6

Do you have any idea on the frequency of visits by hummingbirds to your plants and flowers in the free-ranging pollination experiments depicted in Figure 1? Was the same flower getting visited more than once? (lines 108-117)

* ☒ Addressed by removing text.

**Response**

We elected to remove those data from this version of the manuscript in order to maintain the focus on the experiments in which we standardized the number of floral visits. Based on data in Gannon et al. (2018), open flowers tend to receive multiple visits per day.

### 1.7

It would be nice to know the number of pollen grains deposited on a stigma both for hand pollinations, open-pollinations (Fig. 1), and following visits by birds to hand-pollinated flowers. Having this information might help elucidate pollination mechanisms

* ~~[ ] Addressed in lines~~

**Response**

We wholeheartedly agree that it would be nice to have these data, but we have not had success in collecting them. First, quantification of the number of pollen grains we placed on the stigma during hand-pollination treatments does not seem feasible in the field. The pollen grains are too small to count with a hand lens. Second, we have not had success quantifying pollen grains on the stigma the day after pollination due to problems with molding and potentially pollen loss during transport back to the lab.

### 1.8

Similarly, it would be nice to know the degree to which pollen tubes translate into seeds.

* ~~[ ] Addressed in lines …~~

**Response**

Unfortunately, we have not evaluated this in the current study, though it is an experiment we hope to complete in the future. However, Betts et al. (2015) report a strong correlation between fruit size (a proxy for seed set) and pollen tubes from the same flowers that were left open to free ranging pollinators (r = 0.91, n = 9, P = 0.0003). In the future, we plan to estimate the probability that any given pollen tube results in a seed.

### 1.9

The mean number of pollen tubes in this experiment is exceedingly small (0 to 1). One would think with hand pollination that one would get maximum pollen tube growth, but that doesn’t happen, which makes Heliconias so interesting. I’d be interested to know whether the number of pollen tubes increases with the number of natural visits by hummingbirds (as well as pollen donors).

* ~~[ ] Addressed in lines …~~

**Response**

This is an interesting idea, but is perhaps beyond the scope of this manuscript. One of the major challenges with obtaining data such as these is that it is not an easily manipulated system. Ideally, we would create different pollen pools with varying numbers of pollen donors and supply these different pools to stigmas by hand, but hand pollinations are notoriously unsuccessful with *Heliconia* species. These are worthwhile experiments for the future.

### 1.10

I also wonder whether the low mean number of pollen tubes and the design of the study makes it difficult to detect differences, if they exist.

* [x] Addressed in lines 384-386.

**Response**

The low mean numbers of pollen tubes likely does make detecting differences difficult, but that is part of the reason we find the consistency of the results from the aviary experiments with *H. tortuosa* so compelling. The study design could be strengthened by conducting these experiments in large, naturally occurring populations of these plants, but that would require travel to and research in multiple countries. Given the logistical (and not to mention monetary) challenge of that approach, we opted for conducting these experiments in a living collection where multiple species can be targetted at once. We assert that our approach is conservative with respect to identifying potential species with pollinator recognition, and now point this out in the discussion (lines 384-386). Nevertheless, we hope the reviewers agree that this is a good first step towards testing the generality of pollinator recognition.

### 1.11

Did birds remove nectar from flowers in the field and aviary experiments, and if so, how much?

* [x] Addressed in lines 351-353.

**Response**

Because these flowers have inferior ovaries, measuring the nectar remaining in the flower after a visit requires destructively sampling the flower, precluding the scoring of pollen tubes in that flower. Betts et al. (2015) do report on nectar removal from *H. tortuosa* by various bird species, but we were unable to spare extra flowers to check the remaining nectar in each plant species after visits from each hummingbird species. However, we can confirm the birds removed at least some nectar based on recorded video of visits in which one can see the birds drinking. We have submitted video files to be included in an online supplement.

### 1.12

…while short-billed hummingbirds may be able to remove a small amount of nectar from the flowers of this plant in aviary experiments, they may not be major visitors to this plant in native systems due to competitive exclusion by long-billed hummingbird species that are more effective nectar removers. This has major implications for the pollinator recognition hypothesis as a pollinator filter, because it would suggest that competition with other hummingbird species is the filter, not within-the-tube pollinator recognition as implied by Betts et al. 2015.

* [x] Addressed in lines 317-324, 325-334, 351-353, and 376-383.

**Response**

This is an excellent comment and we agree that pollinator recognition would not be an effective pollinator filter if competitive exclusion limits visitation by hummingbird species with mismatched bill shapes. In a separate project, authors M.G. Betts, A.S. Hadley, and others collected visitation data for *H. tortuosa*, which appears to be largely visited by well-matched green hermit hummingbirds (*Phaethornis guy*). However, birds with mismatched bill shapes still compose ~10% of honest visits. This proportion of visits from species with mismatched bill shapes likely increases in fragmented patches where *P. guy* is much less common (Kormann et al. 2016, Hadley et al. 2018). Furthermore, the proportion of individual birds with mismatched bill shapes that carry *Heliconia* pollen is comparable to the proportion of individual birds with well matched bill shapes that carry *Heliconia* pollen (Betts et al. 2015). This is consistent with our hypothesis that pollinator recognition could act as a pollinator filter since exploitation barriers and competition for resources among hummingbird species may not perfectly sort pollinators according to morphology.

While literature on the pollination of the other *Heliconia* species is limited, we included some text on the potential for a pollinator recognition mechanism to filter pollinators for each plant species based on available data.

## Reviewer 2

**General comments**

Overall, the paper is well written and it is enormously important to also present equivocal results as here. I would appreciate more transparency in the methods, however, since much of the methodology is very brief and it is hard to judge for the reader what experiments were actually conducted in how many flowers per species and so on. Further, while the introduction reads smoothly overall, the discussion would benefit from some reworking - it is lengthy in parts. I appreciate, however, that the authors touch upon such a complex topic and also take the time to discuss shortcomings and limitations.

**Response**

We thank the reviewer for the commendations on our efforts to present some equivocal and some confirmational results from a study in which we reproduced previous experiments with *Heliconia tortuosa* and expanded to other taxa. We hope the reviewers find our Methods and Discussion sections improved.

### 2.1

79 - check this sentence, repeats the same statement

* ☒ Addressed in line 79.

### 2.2

85 - I think it would be important to add ideas about purity of pollen loads pollinators carry - would you expect those to differ (i.e. precision in size matching, deposition on safe sites, depositing on different areas of the pollinators body…) - you cited Muchhala before, so this would tie in easily here

* [x] Addressed in lines 335-340.

We appreciate the importance of the purity of pollen loads and placement of pollen on pollinator bodies, but thought that introducing these ideas in the introduction interrupted the flow of the writing. We therefore chose to include some text on this critical component of pollination later in the manuscript.

### 2.3

132f - as it is written now, it is hard to follow all the different experiments you have done; I suggest you break this long paragraph into smaller paragraphs and include details on sample sizes (species, how many individuals per species) in each paragraph; also, I saw that you have longer experimental procedures as supplement, but I didn’t see them being cited in the text? Anyways, I would suggest you include some more details still in the main text so that the reader can more easily understand what experiments you conducted.

* ☒ Addressed in lines 99-191.

**Response**

In response to this comment, we have rewritten the methods section in a way we hope will improve tranparency. The revised text includes references to a supplement with greater experimental detail, including photographs of the aviaries and hand-pollination techniques.

### 2.4

138 - how did you standardize quantity? This may still differ, no matter whether the pollen came from the same donor. Also, there is no explanation of how you applied the pollen to the stigmas. Furthermore, you do not specify anywhere whether flowers were emasculated before? If not, how can you rule out that pollinators did not remove pollen grains when visiting?

* ☒ Addressed in lines 167-173.

**Response**

These are all good points and we included greater detail in the methods section in order to clarify them. First, we did not emasculate the flowers because, in prior experience, flower damage has proven detrimental to pollen tube development, even in flowers left open to free-ranging pollinators. Second, the reviewer is correct that we were unable to perfectly standardize the number of pollen grains at the stigmatic surface due to the small size of *Heliconia* pollen. Instead, the same experimenter (author D.G. Gannon) hand pollinated each flower using a 20x hand lens to ensure that an even layer of pollen was spread across the stigma. There is indeed variability in the number of grains adhered to the stigmatic surface across these trails, but we attempted to limit this variability as much as possible.

While we cannot rule out that hummingbirds removed pollen from the stigma (in fact we think this did occur and resulted in the reduced pollen tube numbers in *H. wagneriana* flowers that were visited by hummingbirds), we detail a number of reasons why we do not expect this to underly the increased number of pollen tubes in *H. tortuosa* and *H. rostrata* flowers visited by morphologically matched hummingbirds.

### 2.5

146 - could hummingbirds dislodge pollen grains from the stigmas when visiting?

* ☒ Addressed in lines 295-304.

This is a good question, and we do expect that some pollen may be dislodged by hummingbirds. In our experience, *H wagneriana* pollen dessicates quickly compared to the other species and often fails to adhere to the stigmatic surface. We therefore expect this could have had a greater effect on the results for *H. wagneriana* than the other species. However, because the flowers were not emasculated, we expect that pollen dislodged by hummingbirds would be replaced on the stigma by self pollen due to the lack of separation between anthers and stigma (Figure 1).

### 2.6

150 - please include per species sample sizes

* ☒ Addressed by adding Table 1.

We have taken care to add a table with species-by-treatment sample sizes to the manuscript.

### 2.7

159 - I do not fully see how this test really allows you to control for potential pollen-rearrangement by hummingbirds. The surface of a pipette tip is different from feathers (i.e. even more electrostatically charged); is it possible to insert the pipette tip into the flower in the same angle/way a hummingbird would insert its bill?

* ☒ Addressed in lines 304-306.

**Response**

We thank the reviewer for giving this careful thought and consideration. Our point here was not to say that we “controlled” for the potential of pollen rearrangement by hummingbirds with these experiments. Instead, we aimed to justify why we think this mechanism is not very likely given that inserting a pipette tip into the flower, which we can do in a manner similar to a hummingbird, could also rearrange pollen grains on the stigma. We have edited the language in the new version of the manuscript in an attempt to make this clearer.

### 2.8

253 - is there any previous experimental evidence whether the different hummingbird species differ in their pollination efficiency under natural conditions (i.e. visitation frequency and seed set)? This would be important information to add here in order to judge the relevance of your finding, i.e. whether increased pollen germination following the visit of a specific species may actually occur naturally.

* ☒ Addressed in lines 317-324, 325-334, 354-362, and 376-383.

This is an excellent comment and something brought up by both the editor and another reviewer. Please see section 1.12 for our response to the same comment made by another reviewer.

### 2.9

273 - I think this is information that should come earlier, i.e. whether the different Heliconia species differ in flower size and may hence be expected to be adapted to a certain subgroup of hummingbirds

* ☒ Addressed in lines 133-143.

In the revised version of the manuscript, we have included more information on the natural history and morphology of the plant species we tested as well as our expectations with regards to pollinator recognition. We thank both reviewers and the editor for these suggestions.

### 2.10

308 - did you study H. tortuosa at the same locality in both studies? Could it be that it is adapted to different pollinator subsets in different populations?

* [x] Addressed in lines 109-111.

**Response**

For both the 2015 paper and this manuscript, we studied *H. tortuosa* plants in the same region. While testing whether *H. tortuosa* is adapted to different pollinators in different geographic locations (and with differing degrees of fragmentation, (Hadley et al. 2018)) is beyond the scope of this manuscript, it is an intriguing idea that we have been considering for future work.

### 2.11

Fig 2 - it would be helpful to include the images of the flowers here as well, so that the reader can judge him/herself whether bill shape and flower shape relate…

* ☒ Addressed in Figure 1

We thank reviewer 2 for this excellent suggestion. We added a panel to Figure 1 (Figure 2 in the original version) so the reader can compare bill and flower shapes across species.

**References**

Betts, M. G., A. S. Hadley, and W. J. Kress. 2015. Pollinator recognition by a keystone tropical plant. Proceedings of the National Academy of Sciences 112:3433–3438.

Hadley, A. S., S. J. K. Frey, W. D. Robinson, and M. G. Betts. 2018. Forest fragmentation and loss reduce richness, availability, and specialization in tropical hummingbird communities. Biotropica 50:74–83.

Kormann, U., C. Scherber, T. Tscharntke, N. Klein, M. Larbig, J. J. Valente, A. S. Hadley, and M. G. Betts. 2016. Corridors restore animal-mediated pollination in fragmented tropical forest landscapes. Proc. R. Soc. B 283:20152347.

Morrison, B. M. L., and C. D. Mendenhall. 2020. Hummingbird–Plant Interactions Are More Specialized in Forest Compared to Coffee Plantations. Diversity 12:126.

Peralta, G., D. P. Vázquez, N. P. Chacoff, S. B. Lomáscolo, G. L. W. Perry, and J. M. Tylianakis. 2020. Trait matching and phenological overlap increase the spatio-temporal stability and functionality of plant–pollinator interactions. Ecology Letters 23:1107–1116.

Solís‐Montero, L., and M. Vallejo‐Marín. 2017. Does the morphological fit between flowers and pollinators affect pollen deposition? An experimental test in a buzz-pollinated species with anther dimorphism. Ecology and Evolution 7:2706–2715.

Temeles, E. J., J. Liang, M. C. Levy, and Y.-L. Fan. 2019. Floral isolation and pollination in two hummingbird-pollinated plants: the roles of exploitation barriers and pollinator competition. Evolutionary Ecology.