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# Limited reproductive interference despite high rates of heterospecific pollen transfer among co-occurring bat-pollinated *Burmeistera*

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## Abstract

**Premise:** Many tropical plants are bat-pollinated, but these mammals often carry copious, multispecific pollen loads making bat-pollinated plants susceptible to heterospecific pollen deposition and reproductive interference. We investigated pollen transfer between sympatric bat-pollinated *Burmeistera* species and their response to heterospecific pollen deposition from each other.

**Methods:** We quantified conspecific and heterospecific pollen deposition for two populations of *B. ceratocarpa*, a recipient species in heterospecific pollen transfer interactions, that co-occur with different donor relatives (*B. borjensis* and *B. glabrata*). We then used a cross-pollination scheme using pollen mixtures to assess the species' responses to heterospecific pollen deposition in terms of fruit abortion and seed production.

**Results:** *Burmeistera ceratocarpa* received significantly more heterospecific pollen from its relatives at both sites than its own pollen was deposited on its relatives. However, heterospecific pollen deposition only affected seed production by *B. borjensis* and *B. glabrata*, but not by *B. ceratocarpa*, suggesting that early acting post-pollination barriers buffer the latter against reproductive interference. Crosses between sympatric and allopatric populations suggest that the study species are fully isolated in sympatry, while isolation between allopatric populations is strong but incomplete.

**Conclusions:** We did not observe evidence of reproductive interference among our study species, because either heterospecific pollen deposition did not affect their seed production (*B. ceratocarpa*) or they receive heterospecific pollen only rarely (*B. borjensis* and *B. glabrata*). Frequent heterospecific pollen deposition might favor the evolution of barriers against foreign pollen (as in *B. ceratocarpa*) that alleviate the competitive costs of sharing low fidelity pollinators with co-occurring species.

KEYWORDS

bat pollination, floral fitness, Lobelioideae, pollinator sharing, reproductive isolation, seed production

When shared pollinators alternate foraging visits between co-flowering plants, pollen might be transferred interspecifically (Morales and Traveset, 2008; Ashman and Arceo-Gómez, 2013; Moreira-Hernández and Muchhala, 2019). Heterospecific pollen arriving on a stigma can affect reproduction by preventing successful adhesion and germination of conspecific pollen grains, and if the species are related closely enough, heterospecific pollen might be able to produce pollen tubes that compete or interfere with conspecific ones in the style (Morales and Traveset, 2008; Ashman and Arceo-Gómez, 2013; references therein). Such reproductive interference can have profound evolutionary consequences when it occurs between sympatric relatives. If the interacting species are interfertile, heterospecific pollen deposition will lead to hybridization, which would be maladaptive unless hybrids' fitness equals or surpasses that of the parentals. And if the species are already fully reproductively isolated, heterospecific pollen deposition will lead to fitness costs by diminishing

  

opportunities for successful intraspecific pollination and seed production. In either of these two scenarios, frequent heterospecific pollen deposition is expected to be detrimental for a species and thus favor the evolution of pre- and post-pollination isolation barriers (Moreira-Hernández and Muchhala, 2019). The role of pre-pollination (i.e., pollinator) isolation in preventing reproductive interference has received considerable attention in the literature (Muchhala and Potts, 2007; Huang and Shi, 2013; Armbruster et al., 2014; Whitehead and Peakall, 2014; Kay et al., 2019), but far less attention has been given to post-pollination barriers (Ashman and Arceo-Gómez, 2013; Streher et al., 2020; but see Pérez-Barrales and Armbruster, 2023 [in this issue]).

Post-pollination mechanisms are expected to confer tolerance against foreign pollen and limit reproductive interference by increasing the performance of conspecific pollen or diminishing that of heterospecific pollen in the stigma and style (reviewed by Ashman and Arceo-Gómez, 2013; Moreira-Hernández and Muchhala, 2019). For example, the presence or absence of stigma exudates and the morphology of the surface papillae constitute early-acting barriers that may prevent foreign pollen adhesion and germination. Late-acting barriers take place inside the style during pollen tube growth and elongation; here the stylar tissue can arrest heterospecific pollen tubes and only allow conspecific ones to reach the base of the style. One example of such a barrier occurs when the interacting species differ in their incompatibility system. Pollen from self-incompatible (SI) species is often able to achieve successful fertilization in pistils of self-compatible (SC) relatives, whereas crosses in the opposite direction almost invariably fail, which is known as the SI × SC rule (Goodwillie and Ness, 2013; Harder et al., 1993; Pérez-Barrales and Armbruster, 2023 [in this issue]). This pattern is repeated even within self-compatible species with mixed mating systems, where pollen from outcrossers also outperforms pollen from selfers due to the former being adapted to a wider range of pistil environments (Barrett and Harder, 2017). It has furthermore been hypothesized that similar mechanisms underlying the rejection of self-pollen can also be involved in heterospecific pollen rejection (Ashman and Arceo-Gómez, 2013). The accumulation of pollen–pistil incompatibilities underlying post-pollination isolation mechanisms also require evolutionary time, and thus efficiency of these barriers is expected to increase with phylogenetic distance between the interacting species (Ashman and Arceo-Gómez, 2013; Moreira-Hernández and Muchhala, 2019; Streher et al. 2020). However, tolerance to heterospecific pollen deposition conferred by post-pollination barriers among congeneric species has been observed in a few cases (e.g., *Clarkia*, Arceo-Gómez et al., 2016; *Burmeistera*, Moreira-Hernández et al., 2019; *Silene*, Hao et al., 2023 [in this issue]), suggesting that it might evolve more rapidly under some circumstances. In particular, cases where such barriers are present in sympatry but are absent between allopatric populations (e.g., Kay and Schemske, 2008; Arceo-Gómez et al., 2016) suggest that pollen transfer dynamics in sympatry might favor the evolution of post-pollination barriers to limit reproductive interference or prevent hybridization (Moreira-Hernández and Muchhala, 2019). Moreover, if heterospecific pollen transfer interactions in sympatry are highly asymmetric and result in one species being disproportionally the main recipient, this species could be under strong selection to develop pre- or post-pollination barriers to either avoid or become able to tolerate further reproductive interference from its relatives. Thus, we can expect that, all else being equal and barring significant divergence in pollinator use, species that receive heterospecific pollen more frequently should exhibit higher tolerance than their relatives against negative effects of heterospecific pollen deposition.

Many studies have raised the possibility that plants pollinated by nectar-feeding bats might frequently experience heterospecific pollen deposition given that this group of relatively large and densely furred pollinators commonly carry copious, multispecies pollen loads (Muchhala and Jarrín-V, 2002; Muchhala et al., 2009; Stewart and Dudash, 2016). Not surprisingly, many bat-pollinated species have evolved specialized flowers with elaborate flower morphologies to avoid reproductive interference by depositing pollen on different areas of the bats' bodies (i.e., differential pollen placement; Tschapka et al., 2006; Muchhala, 2008; Stewart and Dudash, 2017). However, it is less clear the extent to which bat-pollinated plants have evolved post-pollination barriers to limit detrimental effects following heterospecific pollen deposition. Our past study with a sympatric species pair of bat-pollinated *Burmeistera* bellflowers (Campanulaceae: Lobelioideae) found differential effects of heterospecific pollen deposition on reproduction between the two focal species (Moreira-Hernández et al., 2019). Using mixtures containing varying degrees of conspecific and heterospecific pollen, we found that *B. ceratocarpa* was still able to successfully produce many seeds under increasing amounts of heterospecific pollen from its congener *B. borjensis*, while seed production in *B. borjensis* significantly decreased with increasing amounts of pollen from *B. ceratocarpa*. Differences in the exsertion of the floral reproductive parts (i.e., exsertion length; Muchhala, 2006) and field experiments suggest that in natural conditions bats transfer pollen predominantly from the long-exserted *B. borjensis* to the short-exserted *B. ceratocarpa* but very little in the opposite direction (Muchhala and Potts, 2007; Muchhala, 2008). Thus, we posited that frequent heterospecific pollen deposition from *B. borjensis* in sympatry might have favored the evolution of strong post-pollination isolating barriers conferring tolerance against negative effects on reproduction in *B. ceratocarpa* (Moreira-Hernández et al., 2019). Testing this idea is the goal of the study presented here.

In this study, we expanded on our previous work (Moreira-Hernández et al., 2019) and investigated whether patterns of asymmetric pollen transfer between sympatric *Burmeistera* species could potentially explain differences in their response to heterospecific pollen deposition from each other. Specifically, our past study showed that the short-exserted *B. ceratocarpa* had a high tolerance to heterospecific pollen deposition from its relative long-exserted *B. borjensis*

(Moreira-Hernández et al., 2019). We hypothesized that this result could be because, in natural conditions, bat pollinators probably transfer pollen from *B. borjensis* to *B. ceratocarpa* more frequently than vice versa, thus promoting the evolution of strong post-pollination barriers in *B. ceratocarpa*. In this study, we quantified rates of pollen transfer and did additional hand-pollination experiments to gain more information on crossing patterns and reproductive interference between these species. First, we measured nightly deposition of heterospecific and conspecific pollen to test whether pollen in fact is transferred asymmetrically between the study species. Second, we did a set of intraspecific pollinations as controls to compare fruit and seed production against our mixed pollinations. Third, we used interspecific crosses between both species to confirm whether they were able to set hybrid seeds. Fourth, we repeated these experiments in a second site where *B. ceratocarpa* co-occurs with a different long-exserted species and predicted that patterns of pollen transfer and post-pollination barrier strength for this species would be similar at both sites. Conversely, we also predicted that the two long-exserted species would receive little pollen from *B. ceratocarpa* at either site and that they would not have evolved strong post-pollination barriers to reduce reproductive interference. Finally, we performed heterospecific crosses between allopatric populations of the study species from both sites. We hypothesized that post-pollination barriers preventing hybridization would have evolved in response to the locally co-occurring species and thus should not affect the success of heterospecific crosses between allopatric populations.

## MATERIALS AND METHODS

### Focal taxa and study sites

The neotropical genus *Burmeistera* H. Karst. & Triana (Campanulaceae; Lobelioideae) comprises ~130 species of terrestrial and hemiepiphytic herbs and shrubs found in cloud forests at middle and high elevations from Guatemala to northern Peru (Lammers, 2007; Knox et al., 2008; Lagomarsino et al., 2014). The highest diversity of the genus is found in Colombia (~80 spp.) and Ecuador (~50 spp.), where cloud forest locations typically harbor one to four (and sometimes up to eight) sympatric *Burmeistera* species (Lammers, 2007; Mashburn, 2019). Flowering overlap between species is extensive; individual plants produce flowers over several months, and population-level flowering occurs all year (Muchhala, 2006). Flowers are zygomorphic (bilaterally symmetrical) and protandrous, with reproductive parts exserted outside of the corolla tube opening by a staminal column (Muchhala, 2006, 2008; Figure 1A–C). At anthesis, the corolla tube opens and anthers release copious pollen from the tip of the staminal column, initiating the male phase, which lasts 24–48 h. The transition to the female phase begins when the stigma protrudes from inside of the staminal column expanding outward and pushing off any remaining pollen (thus preventing self-pollination; Muchhala, 2006). During the female phase, the stigma surface changes from wet, bright, and smooth for the first couple of days to dry, dull, and withered before flowers are eventually shed. The majority of *Burmeistera* species are pollinated primarily by bats, with hummingbird pollination restricted to a handful of species (Muchhala, 2006; Lagomarsino et al., 2017; Figure 1D–F). Fruits in the genus contain thousands of small seeds and are either fleshy or inflated, hollow berries (Lagomarsino et al., 2014; Gamba et al., 2017).

Fieldwork was carried out in two cloud forest locations in northeast Ecuador. The first, Yanayacu Biological Station (0°36′ 03″S, 77°53′22″W; hereafter Yanayacu) is a private biological reserve located at ~2100 m a.s.l. within the Cosanga River valley and close to the small town of Cosanga. The station borders the much larger Antisana Ecological Reserve (1200 $km^2$) and supports a mosaic of abandoned pastures and secondary growth with mature cloud forest found in the upper parts of the property along ridgetops. At this site, we studied the long-exserted species *B. borjensis* and the short-exserted *B. ceratocarpa* (Figure 1A–B), which are common in the forest understory and occasionally along forest edges. In Yanayacu, the exsertion length of *B. borjensis* is 24.5 ± 2.7 mm ($N = 18$) and that of *B. ceratocarpa* is 16.6 ± 0.8 mm ($N = 12$). The second location, Cordillera de los Guacamayos (0°37′22 ″S, 77°50′26″ W; hereafter Guacamayos), is a forested mountain ridge at approximately 2250 m a.s.l. within the Antisana Ecological Reserve. Although this site is located only ~5 km on a straight line from Yanayacu, it is found on the Amazon-facing side of the slopes bordering the Cosanga River valley to the east; thus, it is much more humid and has a strikingly different forest composition (J. Moreira-Hernández and N. Muchhala, personal observations). At Guacamayos, we studied a second *B. ceratocarpa* population and the sympatric *B. glabrata* (Figure 1B, C), which replaces *B. borjensis* as the local long-exserted species. The main accessible trail goes through tall, mature cloud forest where *B. glabrata* and *B. ceratocarpa* are very common along the trail and on small forest gaps. At this site, *B. glabrata* flowers have an exsertion length of 23.3 ± 1.8 mm ($N = 12$), whereas those of *B. ceratocarpa* measure 15.7 ± 0.5 mm ($N = 15$). Flowers of all four populations of the three study species are bat-pollinated and are similar for most floral traits other than exsertion length and the size and shape of the calyx lobes (Figure 1A–C). Also in Guacamayos and Yanayacu are *B. sodiroana* and *B. succulenta*, which are both short-exserted. Although they likely also interact with our focal species via interspecific pollen transfer, they are far less abundant in either location in both the total number of individuals and thenumber of flowers per individual (J. Moreira-Hernández, personal observations).

### Estimating conspecific and heterospecific pollen deposition by bat pollinators

We quantified conspecific and heterospecific pollen deposition by bat pollinators on stigmas of the study

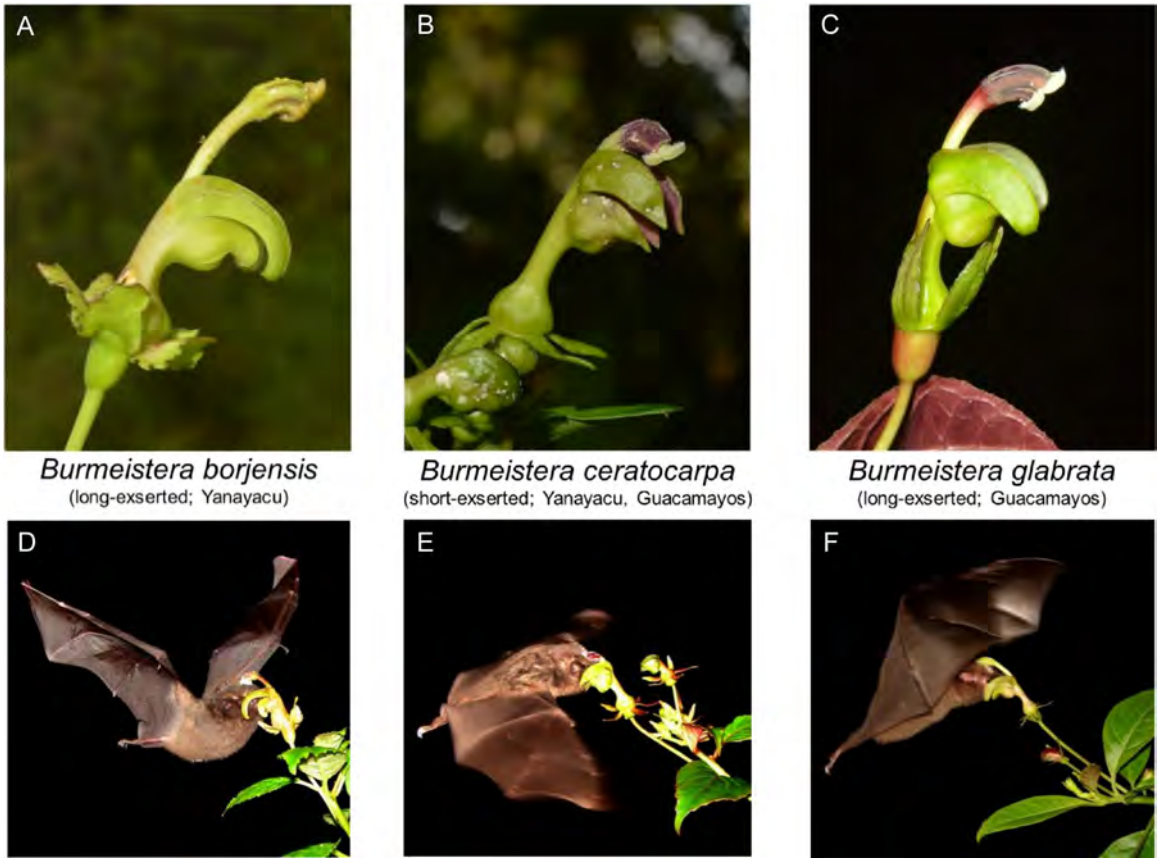


**FIGURE 1** (A–C) Flowers of three bat-pollinated *Burmeistera* species from the two study locations in Ecuador. *Burmeistera* flowers have reproductive structures located on a staminal column exserted outside of the corolla tube, resulting in localized pollen deposition on the head of its bat pollinators. Species like *B. borjensis* and *B. glabrata* (A, C) have a long staminal column that allows their reproductive structures to contact a large area of the bat's head so that pollen is placed on top of the head and upper back; species such as *B. ceratocarpa* (B) have shorter columns, and pollen is placed between the eyes and tip of the snout. (D–F) Nectar-feeding bats *Anoura caudifer* (D, E) and *A. cultrata* (F) visiting the flowers of *B. borjensis*, *B. ceratocarpa*, and *B. glabrata*, respectively. Photos (A-C) by J.I.M.H. and (D-F) by N.M.

species at both sites using methods previously developed for *Burmeistera* (Muchhala, 2003, 2006). Staminal columns of flowers in the field were wrapped with a thin layer of parafilm and a 0.5 × 0.8 cm rectangle of clear double-sided tape was placed at the tip of the column where the stigma is located. These tapes thus collect pollen that wild-foraging bats bring to the flowers. After 24 h, we collected the tape samples, placed them on microscope slides, and covered them with clear single-sided tape. Previous data showed that diurnal pollen deposition by hummingbirds is negligible (Muchhala, 2006), thus, even though the tapes were left for 24 h on the flowers we expect that the pollen samples primarily reflect nightly pollen deposition by bats during the first 8–12 h. Pollen samples were stained with fuchsin gelatin cubes and observed with a light microscope to identify and count all pollen found along two perpendicular transects passing through the center of the tape sample. The stained pollen grains could be identified to species due to differences in grain size and the shape of the colpi between the long- and short-exserted species pair within each study site (Muchhala and Potts, 2007). Pollen counts from our tape samples allowed us to estimate conspecific pollen deposition per stigma for each study species and heterospecific pollen deposition from the other member of the species pair at each of our two study sites.

## Reciprocal cross-pollination experiments

We used a fully reciprocal mixed pollination scheme to study the effect of heterospecific pollen deposition on fruit and seed production in each sympatric *Burmeistera* species pair (i.e., *B. glabrata* and *B. ceratocarpa* in Guacamayos; *B. borjensis* and *B. ceratocarpa* in Yanayacu). We selected 14–25 focal plants from each species at each site choosing individuals with many open flowers and buds for the experiments. Other individuals were also used opportunistically as pollen donors. We made pollen mixtures using four fresh male flowers from the same site, varying the ratio of flowers used from each type to make mixtures approximating different relative amounts of heterospecific and conspecific pollen. For example, a pollen mixture made using one *B. borjensis* flower and three *B. ceratocarpa* flowers had a 1:3 ratio of heterospecific:conspecific pollen for pollinating *B. ceratocarpa*. Conversely, the same

mixture could be used as a 3:1 mixture for pollinating *B. borjensis*. These pollen mixtures were then used in sympatric crosses between the species pair in each location. We used four pollen mixture ratios as treatment levels corresponding to increasing heterospecific pollen presence in each mixture: 1:3, 2:2, 3:1, and 4:0 (i.e., a pure heterospecific mixture). We also made pure conspecific (0:4) pollen mixtures as controls using four flowers from other conspecific individuals of the same population. Because these pollen ratios are approximations and not actual known quantities, throughout this study we refer to our treatments as ratios of heterospecific to conspecific flowers used in each mixture. Finally, we also performed pure interspecific pollinations between allopatric populations of the study species to evaluate whether heterospecific pollen from non-co-occurring relatives resulted in fruit and seed production. In these allopatric crosses, we pollinated *B. glabrata* and *B. borjensis* using pollen from *B. ceratocarpa* from the population in the opposite location (i.e., Yanayacu for *B. glabrata* and Guacamayos for *B. borjensis*). Similarly, for each *B. ceratocarpa* population, we used pollen from the respective long-exserted species that was allopatric (i.e., *B. glabrata* for *B. ceratocarpa* from Yanayacu and *B. borjensis* for *B. ceratocarpa* from Guacamayos).

For the experiments, we used 14–25 individual plants per species at each site and 2–3 flowers per plant (Guacamayos: *B. glabrata*: 25 plants and 2.0 ± 0.8 flowers/plant; *B. ceratocarpa*: 14 plants and 2.4 ± 0.9 flowers/plant; Yanayacu: *B. borjensis*: 16 plants and 2.9 ± 2.1 flowers/plant; *B. ceratocarpa*: 16 plants and 2.4 ± 1.6 flowers/plant; means ± SD in all cases). Treatments were assigned to plants at random and, whenever possible, we applied different treatments within individual plants alternating with controls. We were also careful to never use self-pollen in any pollen mixtures applied to a particular stigma. The experiments at Yanayacu for the 1:3, 2:2, and 3:1 mixed pollination treatments were done during field seasons in 2014 and 2017 (Moreira-Hernández et al., 2019); the pure conspecific, pure heterospecific, and allopatric crosses in Yanayacu and all replicates from Guacamayos were done between January and March 2019. During the first set of experiments in 2014 and 2017 at Yanayacu, treatments were applied to female flowers early in the evening only if visual inspection with a hand lens indicated that pollen had not been deposited on the stigma. Bats deposit hundreds of pollen grains per visit (Muchhala, 2003) which changes the stigma appearance from shiny to a matte, dusty look (J. Moreira-Hernández and N. Muchhala, personal observation). Thus, after careful examination, we assumed that shiny, bright stigmas from flowers had just entered female phase and were free of pollen. We did not use flowers whose stigmas had any pollen grains on them or were not shiny and bright. For all other experiments that we did in both locations in 2019, we bagged flowers nearing the end of male phase, precluding the need to visually examine the stigma for previously deposited pollen.

To apply the pollen mixtures to flowers, we used dry bat skins stuffed with cotton that were prepared using standard procedures for mammal specimens in biological collections (Hall, 1962). We simulated pollen deposition by bats by placing the mixture in the respective area of the bat head that would contact each type of flower (i.e., the tip of the snout for *B. ceratocarpa* and the forehead for *B. glabrata* and *B. borjensis*) and then applied it to stigmas early in the evening. We used two different bat specimens for the experiments, and every night each one was used for only one pollen mixture type combination. Specimens were reloaded with pollen mixtures before every pollination and were thoroughly cleaned of pollen with clear tape at the end of the evening. We believe that this method of pollen application reflects the large amount of pollen bats carry on their fur and deposit in natural conditions (Muchhala, 2003; Muchhala and Thomson, 2010). Following each pollination, we covered the flowers to prevent any further pollen deposition by floral visitors. We then marked and labeled the flower pedicel and the subjacent branch node with tape. We revisited the plants after 5 weeks to ascertain fruit fate (matured, aborted, or lost), and mature fruits were collected in 70% v/v alcohol and transported to the lab to estimate total seed production per fruit.

## Statistical analyses

To test for differences in conspecific and heterospecific pollen deposition between the study species and to determine the effect of increasing heterospecific pollen deposition on fruit and seed production, we used generalized linear mixed models (GLMMs) implemented in the R package glmmTMB (Brooks et al., 2017) using R version 4.2.2 (R Core Team, 2021). For each species pair at each site, we modeled the number of pollen grains per tape sample (our stigma proxy) over 24 h using a negative binomial distribution with species and pollen type (conspecific or heterospecific) as fixed effects. We also used $\chi^2$ tests to evaluate whether the proportion of tape samples that contained at least some heterospecific pollen differed between the pair of species at each site. To assess the effect of different levels of heterospecific pollen deposition on the proportion of aborted fruits for each species pair at each site, we built a binomial GLMM using species and the ratio of heterospecific to conspecific pollen in mixtures as fixed effects in the model. Finally, we tested for the effect of heterospecific pollen deposition on the total number of seeds per fruit by the study species with a negative binomial GLMM specifying species and the ratio of heterospecific to conspecific pollen in mixtures as fixed factors. In all models, the identity of the plant bearing each flower was included as a random factor and the significance of the fixed effects was assessed with likelihood ratio tests. When the effect of the ratio of heterospecific to conspecific pollen was significant, we tested for variation across treatment levels using the Tukey–Bonferroni *P*-value adjustment for multiple comparisons using the R package multcomp (Hothorn et al., 2008).

# RESULTS

## Patterns of congeneric heterospecific pollen deposition by bat pollinators

Quantification of pollen deposition samples revealed distinct patterns of conspecific and heterospecific pollen deposited by wild-foraging bats on stigmas of the study species (Figure 2). The species pair at each location received similar nightly deposits of conspecific pollen grains but different amounts of heterospecific pollen deposition. In Guacamayos, the number of conspecific pollen grains deposited on stigmas for *B. glabrata* and *B. ceratocarpa* was not significantly different (mean ± SD: *B. glabrata*, 109.54 ± 47.27, $N = 46$; *B. ceratocarpa*, 80.47 ± 39.16, $N = 45$; likelihood ratio test: $\chi^2 = 1.97$, $P = 0.241$; Figure 2). On the other hand, heterospecific pollen deposition differed between the species because *B. ceratocarpa* received substantial pollen from *B. glabrata* (mean ± SD: 41.42 ± 29.64, $N = 45$), while *B. glabrata* received very little pollen from *B. ceratocarpa* (mean ± SD: 1.73 ± 3.76, $N = 46$; likelihood ratio test: $\chi^2 = 37.84$, $P < 0.001$; Figure 2). The percentage of samples that contained at least some heterospecific pollen also differed between both species; only 29.9% of *B. glabrata* samples had some *B. ceratocarpa* pollen, while 91.1% of the samples from *B. ceratocarpa* had pollen from *B. glabrata* ($\chi^2$ test: $\chi^2 = 11.93$, df = 1, $P = 0.001$).

At Yanayacu, conspecific pollen deposition was slightly but significantly higher for *B. borjensis* than for *B. ceratocarp*a (mean ± SD: *B. borjensis*, 74.33 ± 40.91, $N = 63$; *B. ceratocarpa*, 45.17 ± 31.83, $N = 63$; likelihood ratio test: $\chi^2 = 24.70$, $P < 0.001$; Figure 2). However, *B. borjensis* received very few *B. ceratocarpa* pollen grains (3.17 ± 6.47, $N = 63$), while *B. ceratocarpa* received a low but significant number of *B. borjensis* pollen grains (12.51 ± 15.11, $N = 63$; likelihood ratio test: $\chi^2 = 19.08$, $P < 0.001$; Figure 2). Once again, the proportion of samples with heterospecific pollen differed between both species, with 23.8% of *B. borjensis* samples and 57.1% of *B. ceratocarpa* samples having some pollen from their respective congener ($\chi^2$ test: $\chi^2 = 8.82$, df = 1, $P = 0.003$).

## Effects of heterospecific:conspecific pollen ratios on female reproduction

We pollinated 333 flowers of both species pairs with at least 10 repetitions per pollination treatment (Table 1). In Guacamayos, we pollinated 99 flowers of *B. glabrata* and 69 of *B. ceratocarpa* across all treatments. In *B. glabrata*, the proportion of aborted fruits was lowest for flowers that were pollinated with pure conspecific pollen (20%) in comparison to flowers pollinated with pollen mixtures (40–53%; Table 1). In *B. ceratocarpa* on the other hand, the proportion of aborted fruits was similar among the conspecific control flowers and those pollinated using pollen mixtures (27–40%; Table 1). In both species, all fruits resulting from pure interspecific pollinations were aborted (Table 1). Contrary to expectations, however, analysis of these rates of fruit abortion showed that pollination treatment did not have a significant effect on the probability of fruit abortion by *B. glabatra* and *B. ceratocarpa* in Guacamayos because neither this factor nor its interaction with the species term were significant (pollination treatment: $\chi^2 = 5.991$, $P = 0.112$; species: $\chi^2 = 0.271$, $P = 0.603$; pollination treatment *x* species interaction: $\chi^2 = 1.090$, $P = 0.780$; Figure 3).

In Yanayacu, we pollinated 98 flowers of *B. borjensis* and 67 flowers of *B. ceratocarpa*. The proportion of aborted fruits in *B. borjensis* across the different pollination

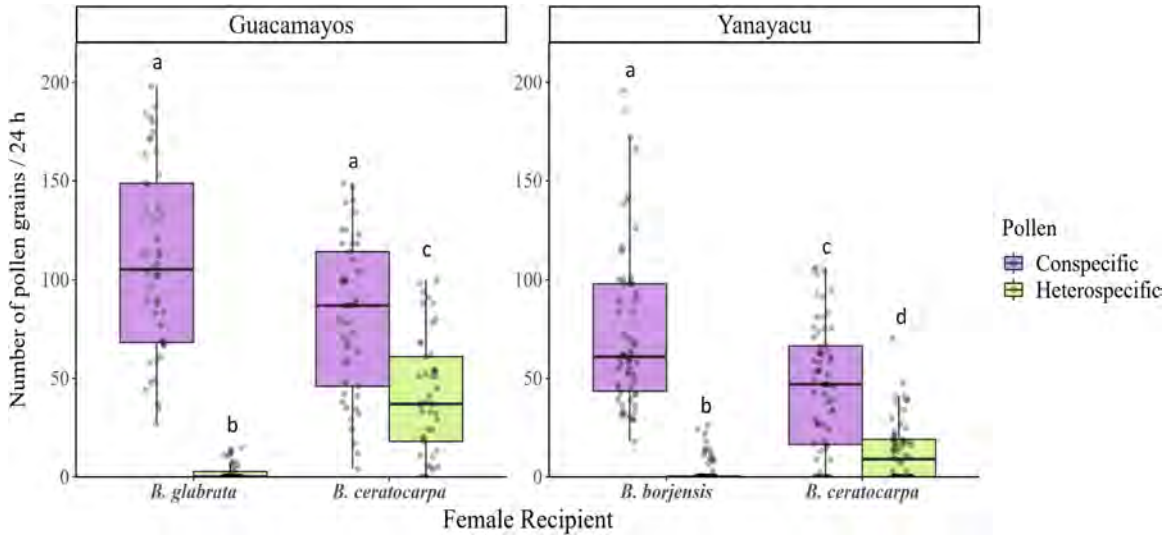


**FIGURE 2** Conspecific and heterospecific pollen deposition over 24 h for two species pairs of bat-pollinated *Burmeistera* (Campanulaceae: Lobelioideae) in two cloud forests in Ecuador. Different letters above boxplots indicate significant differences between species and pollen deposition type at each location after Bonferroni correction for multiple comparisons.

**TABLE 1** Number of hand pollinations performed and fate of the resulting fruits under different ratios of heterospecific (HS) and conspecific (CS) flowers for pollen mixture treatments used in two species pairs of bat-pollinated *Burmeistera* at two sites in Ecuador.

| Site | Species | Ratio of HS:CS flowers in pollen mixtures | Total no. pollinations | Proportion of fruits (*N*) | | |
|---|---|---|---|---|---|---|
| | | | | Matured | Aborted | Lost |
| Guacamayos | *B. glabrata* | 0:4 | 25 | 0.80 (20) | 0.20 (5) | 0 (0) |
| | | 1:3 | 24 | 0.58 (14) | 0.42 (10) | 0 (0) |
| | | 2:2 | 20 | 0.50 (10) | 0.40 (8) | 0.10 (2) |
| | | 3:1 | 15 | 0.47 (7) | 0.53 (8) | 0 (0) |
| | | 4:0 | 15 | 0 (0) | 1.00 (15) | 0 (0) |
| | *B. ceratocarpa* | 0:4 | 20 | 0.65 (13) | 0.35 (7) | 0 (0) |
| | | 1:3 | 12 | 0.67 (8) | 0.33 (4) | 0 (0) |
| | | 2:2 | 11 | 0.64 (7) | 0.27 (3) | 0.09 (1) |
| | | 3:1 | 10 | 0.60 (6) | 0.40 (4) | 0 (0) |
| | | 4:0 | 16 | 0 (0) | 1.00 (16) | 0 (0) |
| *Yanayacu* | *B. borjensis* | 0:4 | 26 | 0.58 (15) | 0.38 (10) | 0.04 (1) |
| | | 1:3 | 24 | 0.50 (12) | 0.50 (12) | 0 (0) |
| | | 2:2 | 18 | 0.61 (11) | 0.39 (7) | 0 (0) |
| | | 3:1 | 15 | 0.53 (8) | 0.47 (7) | 0 (0) |
| | | 4:0 | 15 | 0 (0) | 1.00 (15) | 0 (0) |
| | *B. ceratocarpa* | 0:4 | 18 | 0.72 (13) | 0.28 (5) | 0 (0) |
| | | 1:3 | 12 | 0.83 (10) | 0.17 (2) | 0 (0) |
| | | 2:2 | 10 | 0.90 (9) | 0.10 (1) | 0 (0) |
| | | 3:1 | 11 | 0.64 (7) | 0.27 (3) | 0.09 (1) |
| | | 4:0 | 16 | 0 (0) | 1.00 (16) | 0 (0) |

treatments was 38–50% (Table 1), but 10–28% in *B. ceratocarpa* (Table 1). As with the previous species pair, all interspecific pollinations in both species resulted in fruit abortion (Table 1). Our analyses showed that the probability of fruit abortion was significantly lower for *B. ceratocarpa* than for *B. borjensis* (species: $\chi^2 = 6.925$, $P = 0.009$; Figure 3B). However, pollination treatment had no effect on fruit abortion by either *B. borjensis* or *B. ceratocarpa* (pollination treatment: $\chi^2 = 0.976$, $P = 0.807$; pollination treatment × species interaction: $\chi^2 = 1.483$, $P = 0.686$; Figure 3).

Our analyses showed that pollination treatment had an overall significant effect on seed production (Table 2, Figure 4). Flowers pollinated using mixtures with greater amounts of heterospecific pollen resulted in fruits with fewer seeds (Figure 4). Moreover, the species term and its interaction with pollination treatment were also both significant in our mixed effect model indicating species-specific differences (Table 2). Both *B. glabrata* in Guacamayos and *B. borjensis* in Yanayacu produced significantly fewer seeds in those treatments where pollen mixtures contained high relative amounts of heterospecific pollen from *B. ceratocarpa* (Table 2, Figure 4). Within *B. ceratocarpa*, on the other hand, total number of seeds per fruit was similar across all pollination treatments in both locations regardless of the composition of the pollen mixture that was used (Table 2, Figure 4). Thus, *B. ceratocarpa* seed production was unaffected by the relative amount of heterospecific pollen from either of its congeners in the pollen mixtures that were applied to flowers.

Finally, our allopatric crosses showed that the populations of our study species from both sites are strongly but not completely isolated from each other. Although the proportions of aborted fruits were still very high (>70%), a small number of fruits developed from all four heterospecific crosses between allopatric populations of the study species (Figure 5, Table 3). As mentioned above, all heterospecific crosses between sympatric species resulted in fruit abortion. However, when long-exserted *B. glabrata* and *B. borjensis* were pollinated with pollen from the *B. ceratocarpa* population from the opposite location, a handful of the crosses formed fruits in both species although


**FIGURE 3** Proportion of mature and aborted fruits across the cross-pollination treatments to evaluate the effect of increased heterospecific pollen deposition in fruit abortion rates of two *Burmeistera* species pairs from two sites in Ecuador. Treatments differed in the ratio of pollen from heterospecific to conspecific flowers used to make the pollen mixtures that were applied to flowers.

with fewer seeds than in the conspecific controls (Figure 5, Table 3). The same occurred in *B. ceratocarpa*. Pollinating *B. ceratocarpa* from Guacamayos with *B. borjensis* pollen from Yanayacu produced fruits in two instances (Figure 5, Table 3). Similarly, two times *B. ceratocarpa* from Yanayacu developed fruits after pollinations with pollen from *B. glabrata* from Guacamayos (Figure 5, Table 3). In both cases, fewer seeds were produced than in the conspecific controls (Table 3). Even though the numbers of pollinations and fruits produced were low in all these cases, these results suggest that post-pollination isolation is apparently complete in sympatry but slightly weaker between allopatric populations of the study species.

## DISCUSSION

This study demonstrates that sympatric *Burmeistera* study species experience little reproductive interference from each other and exhibit strong post-pollination isolation because none of the interspecific crosses within sites set fruit. By quantifying natural patterns of pollen deposition from bats and the number of fruit and seed produced after different levels of heterospecific pollen deposition, we showed that post-pollination barriers among the study species were asymmetric, being stronger in *B. ceratocarpa*, the species that most frequently receives heterospecific pollen in natural conditions. *Burmeistera ceratocarpa* experienced substantial heterospecific pollen deposition from its relatives in both of our study sites, yet it was able to attain high fruit and seed production in our hand-pollination crosses even at the highest ratios of heterospecific to conspecific pollen (3:1). Thus, this species seems to have evolved efficient post-pollination isolation mechanisms that limit reproductive interference caused by heterospecific pollen. In contrast, *B. borjensis* and *B. glabrata* rarely receive foreign pollen in nature, and after our hand pollinations, suffered a decrease in seed set at intermediate and high levels of heterospecific pollen deposition. However, it is also worth highlighting that none of the heterospecific crosses between co-occurring species resulted in the production of hybrid seeds; thus, all three species seem to have complete post-pollination reproductive isolation. The fact that heterospecific crosses between the allopatric populations did result in hybrid fruit and seeds in a few cases suggests that such reproductive isolation is probably stronger in sympatry. Taken together, our results support the hypothesis that in *Burmeistera* the

**TABLE 2** Mixed effects model for the total number of seeds per fruit under different pollination treatments in two *Burmeistera* species pairs at two sites in Ecuador. Linear contrasts within each species are shown by the ratios of heterospecific:conspecific flowers used to make the pollen mixtures that were applied to flowers in the treatment levels being compared. Significant differences ($\alpha = 0.05$) between treatment levels are shown in bold.

| **Negative binomial mixed effects model for total number of seeds per fruit** | | | | | | |
|---|---|---|---|---|---|---|
| **Random effects** | | **Variance** | | | | **SD** |
| Plant | | 6.22E-10 | | | | 2.49E-05 |
| **Fixed effects** | | **$\chi^2$** | | **df** | | ***P*** |
| Pollination treatment | | 68.842 | | 3 | | **<0.0001** |
| Species | | 44.459 | | 3 | | **<0.0001** |
| Treatment × Species | | 17.636 | | 9 | | **0.0396** |
| **Contrasts** | | **Estimate** | **SE** | **df** | ***t*-ratio** | ***P*** |
| Guacamayos | | | | | | |
| *B. glabrata* | 0:4–1:3 | 1.028 | 1.103 | 153 | 0.277 | 1.0000 |
| | 0:4–2:2 | 1.649 | 0.198 | 153 | 4.165 | **0.0022** |
| | 0:4–3:1 | 2.473 | 0.392 | 153 | 5.707 | **<0.0001** |
| | 1:3–2:2 | 1.604 | 0.210 | 153 | 3.620 | 0.0150 |
| | 1:3–3:1 | 2.406 | 0.401 | 153 | 5.266 | **<0.0001** |
| | 2:2–3:1 | 1.499 | 0.269 | 153 | 2.256 | 0.4381 |
| *B. ceratocarpa* | 0:4–1:3 | 1.173 | 0.153 | 153 | 1.218 | 0.973 |
| | 0:4–2:2 | 1.428 | 0.209 | 153 | 2.438 | 0.323 |
| | 0:4–3:1 | 1.483 | 0.232 | 153 | 2.515 | 0.280 |
| | 1:3–2:2 | 1.218 | 0.199 | 153 | 1.207 | 0.974 |
| | 1:3–3:1 | 1.265 | 0.218 | 153 | 1.360 | 0.944 |
| | 2:2–3:1 | 1.039 | 0.192 | 153 | 0.206 | 1.000 |
| Yanayacu | | | | | | |
| *B. borjensis* | 0:4–1:3 | 1.396 | 0.153 | 153 | 3.040 | 0.0841 |
| | 0:4–2:2 | 1.286 | 0.142 | 153 | 2.284 | 0.4193 |
| | 0:4–3:1 | 2.183 | 0.308 | 153 | 5.535 | **<0.0001** |
| | 1:3–2:2 | 0.921 | 0.114 | 153 | −0.666 | 0.9997 |
| | 1:3–3:1 | 1.563 | 0.238 | 153 | 2.941 | 0.1087 |
| | 2:2–3:1 | 1.697 | 0.258 | 153 | 3.479 | **0.0237** |
| *B. ceratocarpa* | 0:4–1:3 | 1.091 | 0.161 | 153 | 0.592 | 1.000 |
| | 0:4–2:2 | 1.404 | 0.230 | 153 | 2.071 | 0.566 |
| | 0:4–3:1 | 1.477 | 0.268 | 153 | 2.149 | 0.512 |
| | 1:3–2:2 | 1.287 | 0.225 | 153 | 1.443 | 0.920 |
| | 1:3–3:1 | 1.354 | 0.259 | 153 | 1.583 | 0.866 |
| | 2:2–3:1 | 1.052 | 0.215 | 153 | 0.249 | 1.000 |

frequent receipt of heterospecific pollen has favored post-pollination barriers that limit reproductive interference in sympatry and prevent foreign pollen from affecting pollination by conspecific pollen.

## Heterospecific pollen deposition by wild-foraging bats

Our three study species received similar amounts of conspecific pollen per stigma (Figure 2). However, we observed a high frequency and intensity of heterospecific pollen receipt in *B. ceratocarpa* and very little in either of its long-exserted relatives. Heterospecific pollen transfer between species in the wild are typically asymmetric and often entail greater costs for one or few of the interacting species (Briscoe-Runquist and Stanton, 2013; Randle et al., 2018; Moreira-Hernández and Muchhala, 2019). In the case of *Burmeistera*, field data and experiments have shown that pollen is transferred between species mostly from long- to short-exserted species given that the long-exserted anthers can make contact with a greater surface area of the bats' head, while the short-exserted anthers only touch the distal part of the bat snout during visitation (Muchhala and Potts, 2007; Muchhala, 2008), in line with the pattern we observed amoung our focal species. Thus, short-exserted species such as *B. ceratocarpa* could be under constant exposure to reproductive interference from heterospecific pollen deposition from sympatric long-exserted relatives. Provided this asymmetry is maintained over sufficient evolutionary time, short-exserted species would be under strong selection to develop effective post-pollination barriers to buffer against reproductive interference caused by heterospecific pollen. Another factor that may impact these heterospecific pollen transfer interactions is the population density of the species involved. At both of our sites, *B. borjensis* and *B. glabrata* are much more abundant than *B. ceratocarpa* and thus likely attract more bats to their flowers and deposit more pollen on their bodies. Both floral exsertion and abundance differences could simultaneously cause greater heterospecific pollen transfer to *B. ceratocarpa*, thus imposing selection on this species to limit reproductive interference.

Our results also shed light on the occurrence of heterospecific pollen deposition by wild-foraging bat pollinators. Sympatric bat-pollinated plants frequently differ in where their pollen is placed on a bat's body (Muchhala and Jarrín-V, 2002; Tschapka et al., 2006; Muchhala, 2008; Muchhala and Thomson, 2012; Stewart and Dudash, 2017), but inherent imprecision in the pollination process probably exposes stigmas of bat-pollinated flowers to frequent deposition of foreign pollen (as seen in this study). Tolerance to heterospecific pollen deposition might be an important factor driving the reproductive success of many bat-pollinated plants that would be easy to overlook. Whether tolerance to heterospecific pollen deposition occurs in other bat-pollinated plants as a mechanism to alleviate costs to reproduction deserves more research.

## Heterospecific pollen deposition and fruit and seed production

Our fully reciprocal cross-pollination design revealed the patterns of post-pollination isolation between our

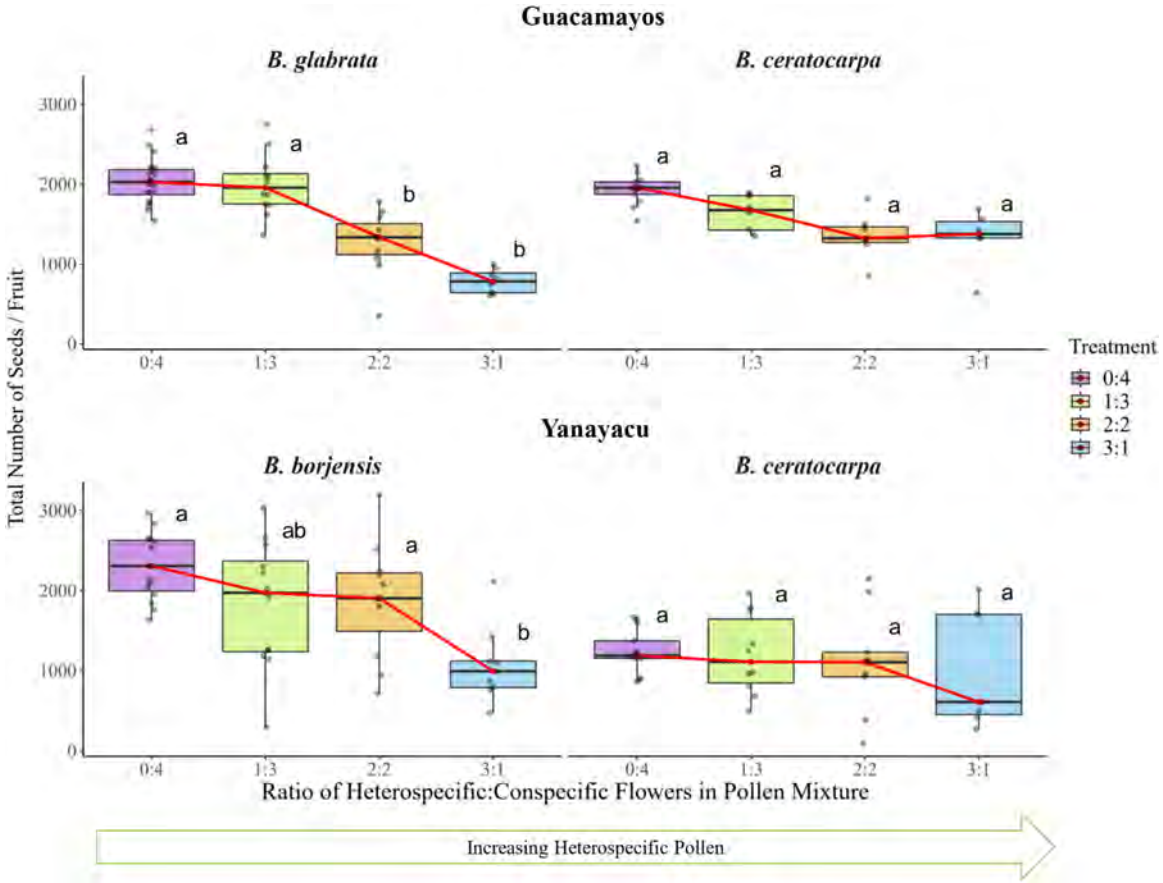


**FIGURE 4** Total number of seeds produced per fruit after different cross-pollination treatments to evaluate the effect of increasing heterospecific pollen deposition on seed production in two of *Burmeistera* species pairs from two sites in Ecuador. Different ratios of pollen from heterospecific to conspecific flowers were used; the proportion of heterospecific pollen increases to the right. Different letters indicated significant differences between treatments within each species after correcting for multiple comparisons ($\alpha = 0.05$). The red line linking the median values across treatments is for visualization purposes only.

*Burmeistera* study species. None of the species produced fruits in sympatric crosses using pure heterospecific pollen, suggesting that they are not hybridizing in sympatry (Figure 3). However, increasing levels of heterospecific pollen deposition revealed species differences that were observed at the stage of seed production. The two populations of *B. ceratocarpa* showed modest but non-significant reductions in seed production with increasing ratios of heterospecific to conspecific pollen: from the 0:4 to the 3:1 treatment mean seed production decreased by 25% in Guacamayos and 17% in Yanayacu (Figure 4). In contrast, *B. borjensis* and *B. glabrata* showed a significant decrease in seed production under high amounts of heterospecific pollen from *B. ceratocarpa*: mean seed production dropped from the 0:4 to the 3:1 treatment by 60% for *B. glabrata* and 57% for *B. borjensis* (Figure 4). Finally, allopatric crosses between our study species using pure heterospecific pollen resulted in low fruit and seed production (Figure 5). Together, these results suggest that (1) our study species exhibit complete post-pollination isolation in sympatry, (2) these isolating barriers are more efficient in *B. ceratocarpa* to the point that even high amounts of heterospecific pollen did not noticeably affect intraspecific pollination, and (3) post-pollination isolation in these species seems to be strong but incomplete between allopatric populations.

Sympatric populations of close relatives are often isolated by post-pollination barriers that limit hybridization. These barriers are often asymmetric, such that the pistil of one species is more successful at arresting pollen germination and pollen tube growth from its congener, than vice versa (Tiffin et al., 2001; Figueroa-Castro and Holtsford, 2009; Natalis and Wesselingh, 2012; Matallana et al., 2016; Moreira-Hernández and Muchhala, 2019). We observed that sympatric pure interspecific pollinations did not lead to fruit and seed production, indicating that post-pollination isolation mechanisms limiting hybridization are at play among our study species. However, though hybridization is being prevented, reproductive interference can still occur if the presence of heterospecific pollen and pollen tubes affect conspecific pollen performance and seed production, as we observed for *B. borjensis* and *B. glabrata* after our mixed pollinations; the deposition of heterospecific pollen was detrimental to seed production in these species even when relatively high amounts of conspecific pollen grains were present. These two species are not often exposed

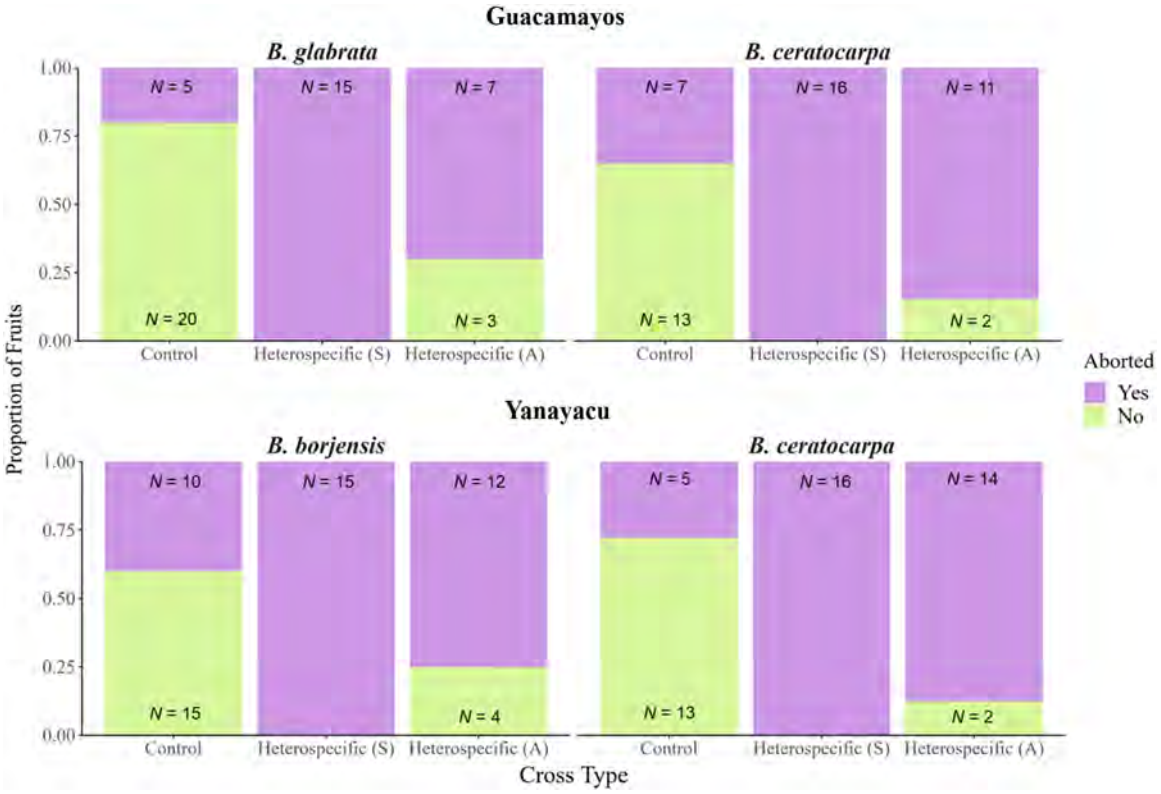


**FIGURE 5** Proportion of matured and aborted fruits from sympatric (S) and allopatric (A) heterospecific crosses in two *Burmeistera* species pairs from two sites in Ecuador.

to this type of reproductive interference, however, because they rarely receive heterospecific pollen in nature. In contrast, heterospecific pollen did not seem to interfere as strongly with conspecific pollen success in *B. ceratocarpa*, because this species was able to produce many seeds across a range of relative amounts of heterospecific and conspecific pollen in the mixtures that were applied to stigmas. This result is strengthened by the fact that the observed ratios of heterospecific:conspecific pollen deposited on *B. ceratocarpa* stigmas by wild, foraging bats per night (between 1:2 to 1:3; Figure 2) were within the ranged tested in our pollination experiments using pollen mixtures (Figure 4). Thus, post-pollination barriers acting in *B. ceratocarpa* pistils seem to prevent reproductive interference, making this species able to tolerate the frequent heterospecific pollen deposition it receives from its sympatric relatives.

Post-pollination reproductive barriers can occur at various stages between pollen deposition and ovule fertilization. Early-acting barriers operate in the stigma or the distal part of the style arresting pollen germination and early pollen-tube growth, whereas late-acting barriers occur farther toward the base of the style and the entrance to ovules, preventing fertilization, or during the post-zygotic phase, causing abnormal embryo development, hybrid seed abortion, or inviability (Ashman and Arceo-Gómez, 2013; Moreira-Hernández and Muchhala, 2019; Coughlan, 2023). Thus, early-acting barriers are more effective at limiting reproductive interference because as heterospecific pollen germinates and the tube grows down the style, the opportunities for negatively affecting conspecific pollen success increase (Ashman and Arceo-Gómez, 2013). We suspect that stigmas of *B. ceratocarpa* are able to arrest foreign pollen germination early and thus allow conspecific pollen tubes to grow down the style unobstructed by heterospecific pollen tubes. This possibility is consistent with our observation that seed production did not vary across pollination treatments in *B. ceratocarpa*, even when the ratios of heterospecific to conspecific pollen in mixtures were roughly equal or even greatly skewed toward the heterospecific (e.g., 2:2 and 3:1; Figure 4). On the other hand, lack of early-acting barriers in *B. borjensis* and *B. glabrata* could have allowed heterospecific pollen tubes to grow down the style and clog the stylar tissue, interfering with conspecific pollen tube performance. This scenario also is consistent with the fact that seed production was reduced in *B. borjensis* and *B. glabrata* only when intermediate and high relative amounts of heterospecific pollen were applied in mixed pollinations. Another observation is that aborted hybrid seeds were very rare (J. Moreira-Hernández, personal observation), indicating a limited role of post-zygotic barriers among the species studied. Thus overall, our results suggest that post-pollination isolation acts early in *B. ceratocarpa* before foreign pollen can negatively

**TABLE 3** Results from sympatric and allopatric heterospecific crosses between two species pairs of bat-pollinated *Burmeistera* at two sites in Ecuador.

| Site | Species | Cross type | No. pollinations performed | Proportion of fruits (N) | | No. Seeds Per Fruit (mean ± SD) |
|---|---|---|---|---|---|---|
| | | | | Matured | Aborted | |
| Guacamayos | *B. glabrata* | Sympatric conspecific (control) | 25 | 0.80 (20) | 0.20 (5) | 2038 ± 282 |
| | | Sympatric heterospecific (*B. ceratocarpa* -G) | 15 | 0 (0) | 1.00 (15) | |
| | | Allopatric heterospecific (*B. ceratocarpa* -Y) | 10 | 0.30 (3) | 0.70 (7) | 788 ± 202 |
| Guacamayos | *B. ceratocarpa* | Sympatric conspecific (control) | 20 | 0.80 (20) | 0.20 (5) | 2038 ± 282 |
| | | Sympatric heterospecific (*B. glabrata*) | 16 | 0 (0) | 1.00 (15) | |
| | | Allopatric heterospecific (*B. borjensis* -Y) | 13 | 0.30 (3) | 0.70 (7) | 788 ± 202 |
| *Yanayacu* | *B. borjensis* | Sympatric conspecific (control) | 25 | 0.58 (15) | 0.38 (10) | 2289 ± 406 |
| | | Sympatric heterospecific (*B. ceratocarpa* -Y) | 15 | 0 (0) | 1.00 (15) | |
| | | Allopatric heterospecific (*B. ceratocarpa* -G) | 16 | 0.25 (4) | 0.75 (12) | 626 ± 115 |
| | *B. ceratocarpa* | Sympatric conspecific (control) | 18 | 0.72 (13) | 0.28 (5) | 1238 ± 272 |
| | | Sympatric heterospecific (*B. borjensis*) | 16 | 0 (0) | 1.00 (16) | |
| | | Allopatric heterospecific (*B. glabrata*) | 16 | 0.13 (2) | 0.87 (14) | 310 ± 65 |

interfere with conspecific pollen and pollen tube growth. Such early-acting barriers against foreign pollen do not seem to occur in the other two species, facilitating opportunities for reproductive interference by heterospecific pollen tubes to play out at late stages down the style. Additional pollen tube growth experiments are needed to confirm these interpretations. Another important consideration that could be the basis for a follow-up study is whether the study species' responses to heterospecific pollen deposition could be explained by differences in their degree of self-compatibility as predicted by the SI × SC rule (Harder et al., 1993; Pérez-Barrales and Armbruster, 2023 [in this issue]). Although *Burmiestera* is protandrous, selfing may still potentially occur via geitonogamy; thus, it is possible that species in the genus could range from fully self-incompatible, to partly or fully self-compatible. If *B. ceratocarpa* were self-incompatible to a greater degree than both of its relatives, our results would be concordant with the SI × SC rule because the more-outcrossing species would exhibit tolerance to heterospecific pollen deposition as the theory suggests (Ashman and Arceo-Gómez, 2013; Pérez-Barrales and Armbruster, 2023 [in this issue]).

## Post-pollination isolation between sympatric and allopatric *Burmeistera* populations

One intriguing result of our study was that post-pollination isolation was apparently incomplete between allopatric populations of the study species, in that hybrid seeds were occasionally produced in all four cases, while pure heterospecific crosses between sympatric individuals of the study species failed in all cases (Figure 5). This result could be indicative of increased reproductive isolation following secondary contact; i.e., sympatric populations are expected to evolve stronger isolating barriers than allopatric populations to prevent hybridization (Coyne and Orr, 2004; Kay and Schemske, 2008). An example of this process occurs in the neotropical genus *Costus*, where a pair of species showed strong post-pollination isolation in sympatry, but this barrier was almost absent between allopatric populations, suggesting that avoiding hybridization had been selected for in co-occurring populations (Kay, 2006; Kay and Schemske, 2008). A similar process could be at play in *Burmeistera*, with increased post-pollination isolation being favored in sympatry. It is particularly intriguing in the two cases where the sympatric and allopatric pollen-donor populations are the same species—namely, when *B. borjensis* and *B. glabrata* were the maternal plants, they would not produce seeds with pollen from sympatric *B. ceratocarpa*, but would with pollen from allopatric *B. ceratocarpa*. However, conclusions from these observations must remain speculative because our sample sizes for sympatric and allopatric heterospecific crosses were too small (10–16 in all cases; Table 3) for robust statistical tests.

A useful follow-up test of whether isolation is stronger between sympatric *Burmeistera* populations could be done by performing hand pollinations using mixtures of all possible combinations of sympatric and allopatric conspecific and heterospecific pollen in equal proportions. Such a design would allow a direct assessment of whether sympatric conspecific pollen performs better with sympatric heterospecific pollen than with allopatric heterospecific pollen (Ashman and Arceo-Gómez, 2013; Arceo-Gómez et al., 2016), testing for a potential mechanism that would explain differences in the success of crosses between sympatric and allopatric *Burmeistera* populations. In addition, the performance of allopatric conspecific pollen with both sympatric and allopatric heterospecific pollen would also shed light on whether strong post-pollination isolation is a general phenomenon across populations or whether it manifests only when sympatric conspecific and sympatric eterospecific pollen interact together in the pistil. Another aspect deserving of future study is whether *Burmeistera* populations that do not co-occur with other congeners also show strong post-pollination isolation and are able to tolerate heterospecific pollen deposition. Unfortunately, the majority of *Burmeistera* species occur in assemblages of two to eight species (Muchhala and Potts, 2007; Mashburn, 2019), so single-species locations are rarely found. Of our study species, *B. borjensis* and *B. ceratocarpa* co-occur together with other *Burmeistera* species throughout their known ranges, and only *B. glabrata* is known to occur by itself at elevations below 1000 m a.s.l. in eastern Ecuador (J. Moreira-Hernández and N. Muchhala, personal observation). Examining post-pollination barriers in these *B. glabrata* populations and those of other species that occur by themselves in part of their ranges is crucial to understand the role of reproductive interference via heterospecific pollen transfer dynamics and the evolution of species barriers acting post-pollination in *Burmeistera*.

## CONCLUSIONS

This study adds support to the hypothesis that patterns of pollen movement by shared pollinators can be related to how species respond to heterospecific pollen deposition. In *Burmeistera*, bat pollinators transfer pollen between sympatric species asymmetrically causing some species to receive foreign pollen very frequently while others rarely do so. Constant exposure to pollen from sympatric relatives seems to have facilitated the evolution of strong post-pollination reproductive isolation in this group. For *B. ceratocarpa*, the species that receives the largest amount of foreign pollen, these post-pollination barriers are strong enough to prevent even high amounts of foreign pollen from affecting the success of conspecific pollen. In contrast, two other *Burmeistera* species that do not commonly receive foreign pollen failed to produce many seeds after receiving mixed pollinations with high relative amounts of heterospecific pollen. Post-pollination barriers between sympatric *Burmeistera* thus seem to be asymmetric, but in the opposite direction to pollen transfer between species, with early-acting barriers conferring tolerance to foreign pollen for species that are common recipients. The overall result is that little reproductive interference was observed between the study species, showing that tolerance to heterospecific pollen can ameliorate the competitive costs of sharing low-fidelity pollinators with sympatric relatives. Additional research will help to elucidate whether post-pollination barriers conferring tolerance against foreign pollen are stronger between sympatric populations than between allopatric ones and whether our results are applicable across other *Burmeistera* populations not exposed to high levels of heterospecific pollen deposition from relatives.

## AUTHOR CONTRIBUTIONS

J.I.M.H. and N.M. conceived and designed the study. J.I.M.H., H.G., N.T., and R.Z.C. collected all field and laboratory data. N.M. and N.O. supervised the project and helped with logistics and permits. N.M. and J.I.M.H. secured funding for this project. J.I.M.H. wrote the manuscript with contributions from N.M. All authors commented and agreed on the final version.

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## DATA AVAILABILITY STATEMENT

All the data used for this study is available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.q573n5tp7 (Moreira-Hernández et al., 2023).

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