

RESEARCH ARTICLE OPEN ACCESS

Locked Pollen, Unchanged Buzzes: Bumblebee Foraging Behavior Persists Despite Pollen Restriction in a Poricidal Legume

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Received: 14 December 2025 | **Revised:** 8 September 2026 | **Accepted:** 10 September 2026

Keywords: buzz pollination | foraging behavior | mutualisms | pollen flowers | poricidal anthers | resource variation

ABSTRACT

In plants that offer pollen as their sole floral reward, this resource is finite and nonreplenishable, making such plants useful systems for studying how resource availability across flowers shapes pollinator behavior. Because tropical ecosystems of the Global South harbor exceptionally diverse plant–pollinator interactions, understanding pollinator behavior in these systems is important for understanding the evolution of plant–pollinator relationships. Using the legume *Chamaecrista latistipula*, which offer only pollen as a floral reward, we assessed how pollen availability affects the foraging behavior of its most frequent pollinator, the bumblebee *Bombus morio*. We hypothesized that bees would reduce handling time and increase their vibrational effort as pollen availability decreased. By partially or completely sealing the pores of poricidal anthers, we created a gradient of pollen availability (0%, 36%, 64%, and 100%, with the latter representing an unmanipulated control). We used video and audio recordings to quantify handling time, buzzing time, grooming time, number of vibrational pulses, and vibration amplitude and fundamental frequency during bee visits. We also recorded the number of flowers visited by each bee after it left the focal flower. Contrary to our hypothesis, pollen restriction did not affect the number of vibrational pulses, buzzing time, fundamental frequency, or vibration amplitude. The only detectable response to the floral resource manipulation was a reduction in handling time, which decreased in the 0% pollen treatment, in which bees omitted grooming because no pollen was accessible. Our results indicate that bees follow a stereotyped vibrational routine when foraging on pollen-offering flowers and that this behavior persists even when the immediate reward is reduced or absent. This limited behavioral adjustment may reflect the absence from our experiment of secondary cues left by previous visitors or constraints imposed by fixed behavioral routine required to exploit poricidal anthers.

1 | Introduction

Flowering plants offer a variety of floral rewards, among which nectar and pollen are the most common (Pinheiro et al. 2014; Bronstein 2015). The quantity and quality of these resources vary among flowers due to phenotypic diversity within

natural populations and resource depletion by previous visitors (Harder and Thomson 1989; González-Almansa Laredo et al. 2026). Consequently, pollinators encounter flowers that differ in resource availability, ranging from resource-rich to resource-poor flowers, often as a result of differences in visitation history (Goldberg 2009; Layek et al. 2022). This variation can

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intensify the conflict between the reproductive interests of plants and the foraging interests of pollinators. Plants benefit from maintaining sufficient floral resources to encourage continued visitation as completely depleted flowers may become unattractive to subsequent pollinators (Fowler et al. 2016). In contrast, pollinators are expected to maximize their net foraging gains by balancing resource extraction against the time and energetic costs of exploiting flowers (Baracchi 2019). This evolutionary conflict is especially pronounced in pollen flowers, where pollen simultaneously functions as the floral reward for bee pollinators and the vehicle for male gamete dispersal.

Pollen flowers, which occur in an estimated 6%–8% of flowering plant species, offer pollen as their sole floral reward and are visited primarily by female bees that collect pollen to provision their offspring (Vogel 1978; Buchmann 1983; Cardinal et al. 2018). In these species, pollen removed and consumed by visitors cannot contribute to fertilization, creating a fundamental allocation trade-off for plants (Harder and Wilson 1994; Vallejo-Marín et al. 2009). One evolutionary framework that explains how pollen flowers mediate this conflict is the pollen presentation theory (PPT), which predicts that, when pollinators are abundant and actively groom pollen from their bodies, plants should evolve mechanisms that promote gradual pollen release, thereby maximizing pollen export across multiple visits (Vallejo-Marín and Lundgren 2025; Pankratz and Russell 2026). Poricidal anthers are prominent adaptations for restricting pollen removal because they release pollen through small apical pores and generally require bees to generate vibrations for efficient pollen extraction (Buchmann and Hurley 1978; Vallejo-Marín 2019). Heteranthery can further mediate this conflict by partitioning stamens into feeding and pollinating sets, thereby promoting pollen export while limiting pollen consumption during individual visits (Vallejo-Marín et al. 2009; Vallejo-Marín and Russell 2024). The amount of pollen released per buzz depends on floral morphology and on vibration amplitude, frequency, and duration (De Luca and Vallejo-Marín 2013). Additionally, empirical studies on buzz-pollinated *Solanum* show that each buzz releases only a small fraction of the available pollen, consistent with the gradual pollen release predicted by the PPT (Vallejo-Marín and Lundgren 2025).

Although floral traits largely determine how pollen is presented to pollinators, the amount of pollen remaining for subsequent visitors also depends on the behavior of buzzing bees during each visit (Rowe et al. 2020; Delgado et al. 2023). First, bees remove pollen in a predictable sequence because feeding and pollinating stamens contact different regions of the body during buzzing (Vallejo-Marín et al. 2009). Second, pollen availability progressively declines as females groom pollen from their bodies and transfer it to the corbiculae, specialized pollen-carrying structures located on the hind legs (Thorp 2000; Koch et al. 2017; Marcelo et al. 2026). Third, bees may modify their foraging decisions according to the amount of pollen obtained from flowers by adjusting visit duration or the biomechanical properties of their vibrations or abandoning flowers and patches with consistently low pollen rewards (Russell et al. 2016; De Luca et al. 2018; Vallejo-Marín 2022; Buchmann and Cane 1989; Pankratz and Russell 2026). Together, these behavioral responses indicate that pollen availability is a dynamic property emerging from the

interaction between floral mechanisms controlling pollen release and the decisions made by buzzing bees during foraging.

Despite extensive research on the biomechanics of buzz pollination and the floral mechanisms regulating pollen release, it remains unclear whether buzzing bees actively modify both their within-flower behavior and their subsequent foraging decisions in response to changes in pollen availability. Most previous studies have focused either on floral mechanisms controlling pollen dispensing or on the biomechanics of bee vibrations, whereas the behavioral responses of pollinators to experimentally manipulated pollen availability remain poorly understood. Here, we experimentally tested whether *Bombus morio*, the main pollinator of *Chamaecrista latistipula*, adjusts its pollen-foraging behavior in response to experimentally manipulated pollen availability, achieved by partially or totally restricting pollen release from the poricidal anthers. First, we tested whether bees could visually discriminate between the different stamen morphs, which could influence their initial perception of resource availability. We then experimentally manipulated pollen accessibility by obstructing the anther pores to evaluate whether bees adjust their vibrational behavior, handling time, buzzing time, and grooming in response to reduced pollen release. Additionally, we examined whether changes in pollen accessibility influence postvisit decisions, such as the number of subsequent flowers visited within a patch. Based on PPT, we predicted that bees would adjust both their within-flower behavior and their subsequent foraging decisions according to floral resource availability. Specifically, we hypothesized that under restricted pollen accessibility, bees would spend less time handling flowers and buzzing the poricidal anthers. Additionally, we expected bees to reduce grooming time because of a lower pollen content on their bodies while increasing the relative peak amplitude of vibrations to compensate for difficult pollen release without altering the fundamental frequency of their buzzes. Finally, we predicted that only after visiting flowers with complete pollen restriction (0% pollen availability) would bees subsequently visit fewer flowers or abandon the patch entirely, a response not expected after visits to flowers allowing partial or total pollen removal.

2 | Materials and Methods

2.1 | Experimental Garden Creation

Thirty *C. latistipula* individuals were transplanted from a population located on the banks of the Cantareira reservoir, in the municipality of Franco da Rocha/SP, to the Federal University of ABC, São Bernardo do Campo, SP, in December 2018. The individuals were accommodated in 18-L pots, at the peak of the rainy season, to minimize post-transplant mortality. The soil in the pots was composed of a mixture of the plants' original substrate with 60% fine washed sand and 40% vegetable soil. The 30 transplanted individuals were used in two experiments conducted between February and April 2019, on nonconsecutive days, in an experimental garden at the Federal University of ABC (Figure S1). During the experiments, the plants were kept in an open field, irrigated weekly, and fruits

were removed daily to ensure the continuity of flower production throughout the experimental window.

2.2 | Focal Species and Study Area

We chose the legume *C. latistipula* (Bastos et al. 2025), whose flowers have poricidal anthers (Figure 1A,B), and the bee species *B. morio* (Figure 1C,D), both widely distributed throughout Brazil (BFG - The Brazil Flora Group 2015; Franoso et al. 2018), as our study model. The genus *Chamaecrista* Moench (Caesalpinioideae, Fabaceae) contains between 330 and 367 species (Lewis 2005, Mendes et al. 2021), of which approximately 258 occur in Brazil, mainly in Neotropical savanna areas (Mendes et al. 2021). Species of *Chamaecrista* are predominantly pollen flowers with poricidal anthers and rely on buzz-pollinating bees for pollen removal (Valadão-Mendes et al. 2025). Despite the diversity among *Chamaecrista* species, especially concerning their vegetative characters, there is considerable constancy in floral traits within the group, making studies on the pollination of these species essential for understanding the genus's natural history (Gottsberger and Silberbauer-Gottsberger 1988; Fenster et al. 2004). Recent work has also emphasized the central role of buzz-pollinating bees in determining pollen release dynamics

and floral function in *Chamaecrista* species (Rando et al. 2026). *C. latistipula* is an endemic Brazilian shrub, and its flowers are on average 2 cm in diameter, with five sepals and five petals, one of which is more curved and larger, called the cucullus (Nogueira et al. 2018). The flowers of this species have ten fertile stamens with poricidal anthers, with seven smaller stamens and three larger stamens (Nogueira et al. 2018). These flowers depend exclusively on bee vibrations for pollen release from the anthers and for the transport and deposition of this pollen on the flower stigma (Gottsberger and Silberbauer-Gottsberger 1988; Nogueira et al. 2018; Barbosa et al. 2023). In natural populations, *C. latistipula* plants produce flowers almost all year round, with a peak flowering period between January and March (Nogueira et al. 2018). The experimental area where this study was developed (São Bernardo do Campo – SP; 23°40'40.8"S 46°33'49.0"W; Figure S1) has a seasonal climate, with rainy summers between November and February and dry winters between May and September.

The focal bee species of this study was *B. morio* (Bombini, Apinae), one of the seven *Bombus* species recorded in Brazil (Franoso et al. 2016). Characterized by a robust and completely black body, covered with dense setae that facilitates pollen collection (Cameron et al. 2007), *B. morio* is a social bee species

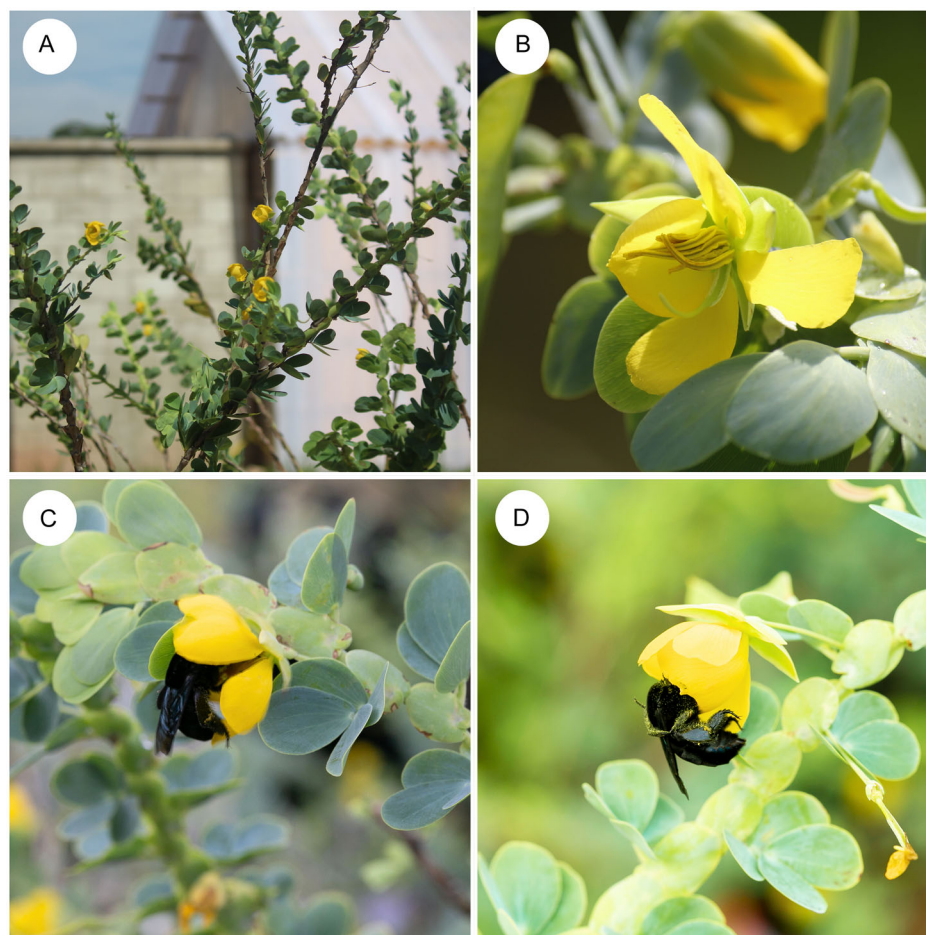


FIGURE 1 | *Chamaecrista latistipula* flowers and visits by *Bombus morio* bees in the experimental garden of the Federal University of ABC. (A) Individuals from the experimental garden where the experiment was conducted. (B) *C. latistipula* flower, focusing on the androecium and poricidal anthers. (C) Detail of the thoracic muscle vibration behavior to remove pollen from the poricidal anthers. (D) Detail of the bees' grooming time during flower visits, noting that the bee is collecting pollen trapped in its upper region to store in its corbicula. Photos by Delgado, T.

of great ecological importance. It is widely distributed in Brazilian savannas, notably in the Cerrado, where it stands out as an efficient pollinator of several *Chamaecrista* species (Gottsberger and Silberbauer-Gottsberger 1988; Nogueira et al. 2018; Fidalgo et al. 2018; Valadão-Mendes et al. 2025). Its relevance is even greater due to the females' behavior of performing buzz pollination, in which they emit vibrations that release pollen from poricidal anthers, a resource inaccessible to many other bees (Cardinal et al. 2018). This technique not only ensures the resource for the colony but also ensures the pollination of plants that strictly depend on this mechanism. Therefore, the vibrational behavior of *B. morio* is crucial for studying the effectiveness of pollen transfer, positioning it as a model species for investigations into plant–pollinator mutualism in the Cerrado.

2.3 | Selective Exclusion of Stamen Morphs

Based on the hypothesis of division of labor, before performing experimental manipulations, we investigated whether bees visually perceive the different stamen morphs distinctly. Considering that *C. latistipula* flowers have seven short stamens and three long stamens (Figure 1B), we tested the visual attractiveness of each group through the selective exclusion of stamen types. For this, we marked flowers from 10 plants and established five experimental conditions: (i) intact flowers with all 10 stamens (control, CO, $N = 11$); (ii) flowers with the removal of the three long stamens (RL, $N = 11$); (iii) flowers with the removal of the seven short stamens (RC, $N = 10$); (iv) flowers with the removal of the three long stamens and four short stamens, leaving only three short stamens (RP, $N = 13$); and (v) flowers with the total removal of all 10 stamens (RT, $N = 11$). After applying each treatment to virgin flowers, we conducted 30-min censuses per flower to record the frequency of visits by *B. morio* bees, with each plant contributing at least one flower in each experimental condition, and observed the duration of the visits.

2.4 | Does Experimentally Restricted Pollen Availability Influence Bee Foraging Behavior on Flowers?

After confirming that bees do not visually differentiate between anther sizes (Figure 2A,B), we proceeded to the central experiment to test our hypothesis that bees adjust their foraging behavior during floral visits in response to restricted pollen availability in *C. latistipula*. To reduce pollen release, we manipulated the opening of the anther pores using quick-drying fabric glue (Acrilex) applied with fine needles. Prior to the main experiment, we conducted a preliminary test to evaluate whether the glue itself could affect bee visitation. For this, glue was applied to a petal, and bee behavior was compared with that observed in unmanipulated flowers (see Figure S2 for details). As no detectable effect on bee behavior was observed, the glue was subsequently used in the manipulative experiment. We then conducted the main experiment following a procedure similar to that of Papaj et al. (2017). We created four different levels of pore

obstruction on the anthers of previously bagged preanthesis buds to protect them from unwanted visits. The treatments, varying the degree of anther pore obstruction, were applied the following morning between 05:00 and 06:00, immediately after flower opening. The treatments systematically applied in each plant comprised four levels of pollen availability: 100% (unobstructed control; $N = 16$), 64% (long-anther obstruction; $N = 16$), 36% (short-anther obstruction; $N = 15$), and 0% (total obstruction; $N = 15$; Table S1). In total, we included 16 plants, with all experimental treatments being applied to each plant. Our experimental design used four different flowers per plant for each level of poricidal anther obstruction/restriction. However, in some cases, we lost flowers from one of the treatment groups, totalizing 62 focal flowers in which the behavior of *B. morio* bees were sampled.

After applying the treatments, we removed the tulle bag from each flower individually and exposed them to a single visit by *B. morio* bees. Immediately after this visit, the tulle bags were repositioned to prevent any subsequent visits, ensuring that each flower was visited only once. For each visit, we recorded bee behavior through filming and sound recordings, and we also observed the number of flowers visited after the focal bee leaves the treated flower. From these bee behavior records, we measured the flower handling time, defined as the interval between the bee's landing and its flight departure (Lichtenberg et al. 2018) and subdivided this period to characterize specific behaviors. Thus, we determined the buzzing time (Kawai and Kudo 2009; Barbosa et al. 2023) as the sum of the periods during which the bees vibrated the poricidal anthers; the total number of vibrational pulses, obtained by sequentially counting these events; and the grooming time (Lichtenberg et al. 2018; Barbosa et al. 2023), which corresponds to the period when the bee removes dispersed pollen from its body, transferring it to the corbícula of its legs. Grooming time was quantified throughout the entire visit, including periods when bees groomed while temporarily leaving the flower, as long as they subsequently returned; therefore, grooming is a component of total handling time. Additionally, we observed the visitation pattern of the population's flowers after the bee visited the focal-treated flower. The *B. morio* visits were filmed with a Canon T5i camera, focused on the flower's poricidal anthers. To process the videos, we used Microsoft Photos (2021) and the "Edit and Create" tool, applying the "Add slow motion" option at the "slowest" speed setting to facilitate the characterization of bee grooming time.

For sound recordings, we used a Sony ICD-PX240 recorder associated with a unidirectional Yoga HT-81 microphone, positioning the microphone 12 cm from the focal flower to standardize sound capture. For each visit, we identified and isolated the segments corresponding to the flower's handling time, the interval between the bee's landing, and the resumption of flight after the visit. Additionally, the vibrational pulses ("buzzes") were counted, and the total buzzing time was determined. All sound recordings were analyzed with Audacity 3.0.5 (www.audacityteam.org). During each visit, bees emitted multiple vibrational pulses to extract pollen grains (e.g., Buchmann 1983, Vallejo-Marín 2022). Therefore, we observed the spectrograms of each "buzz," which were edited using a size of 8192, Hamming window, and maximum frequency of 3000 Hz, displayed in grayscale. To reduce background noise, we applied a high-pass filter with a 12 dB per octave roll-off and a cutoff

frequency of 100 Hz, following the same standards as those in the sound analyses described by De Luca et al. (2019). From the spectrograms generated for all vibrational pulses recorded in each visit, we estimated the peak frequency and relative amplitude. As each bee emitted several pulses per visit, we calculated the average of these values, defining the fundamental frequency (Hz) and the relative amplitude peak (dB) as the averages of the pulses during the floral visit.

Finally, to quantify the degree of pollen restriction in each experimental treatment, we estimated the available pollen in the two anther types (long and short) of *C. latistipula* following the pollen estimation method of Barbosa et al. (2023). For calibration, we sampled 37 flowers from 13 individuals (Table S1). Whole flowers were fixed in FAA for 24 h and subsequently stored in 70% ethanol until analysis. At the time of pollen counting, long and short poricidal anthers were separated from each flower and processed independently. Pollen grains were released by vortex agitation with a steel grinding ball in an aqueous Tween-20 solution, ensuring complete pollen extraction. Subsamples of the homogenized solution were collected during continuous agitation and loaded onto a Neubauer chamber for pollen counting under optical microscopy. Total pollen number per anther was estimated by scaling pollen counts from multiple subsamples to the total solution volume (see Appendix S4 in Barbosa et al. 2023). Per-anther pollen estimates were then used to calculate the effective pollen restriction applied to each flower in the bee behavioral experiments.

2.5 | Statistical Analysis

To test the assumption that anthers are visually perceived distinctly by bees, we quantified both the number of visits performed in 30-min censuses and the average duration of each visit to flowers under different experimental conditions. Initially, we defined the experimental condition as a categorical predictor variable with the following levels: CO (intact flowers), RL (removal of long stamens), RC (removal of short stamens), RP (removal of three long stamens and four short stamens), and RT (total removal of stamens). Then, we developed a generalized linear mixed model (GLMM) with a negative binomial distribution, using the number of bee visits in 30-min censuses as the response variable, and plant identity as a random variable, using the “glmer.nb” function from the lme4 package (Bates et al. 2015). Model assumptions were evaluated using simulation-based residual diagnostics implemented in the DHARMA package (Hartig 2026), including tests for residual dispersion, uniformity, outliers, and zero inflation. No violations of model assumptions were detected. For the analysis of the visit duration response variable, we constructed a linear mixed model (LMM) with a Gaussian distribution, using plant identity as a random variable in the model, using the “lmer” function from the lme4 package (Bates et al. 2015). We tested the assumptions of normality and homogeneity of variance of the residuals through QQ plots and residual plots. As these assumptions were not met, we applied a logarithmic transformation and reran the model. Plant identity was included in the models as a random

variable due to the involvement of repeated measurements of several flowers from the same plant throughout the experiment

To test whether the behavior of *B. morio* bees varies according to pollen restriction treatment on *C. latistipula* flowers, we fitted seven mixed-effects models, one for each behavioral response: five LMMs and two GLMMs. In all models, pollen availability was included as a fixed effect with four categories as 100% (control), 64% (short-anther obstruction), 36% (long-anther obstruction), and 0% (total obstruction). The 100% treatment (control) was set as the reference level. Plant identity was included as a random intercept in all models to account for repeated observations from multiple flowers on the same plant. The continuous response variables (handling time, buzzing time, grooming time, fundamental frequency, and relative amplitude peak) were analyzed using Gaussian LMMs, using the “lmer” function from the lme4 package (Bates et al. 2015). Handling time, buzzing time, grooming time, and fundamental frequency were log-transformed to meet model assumptions. Residual normality and homoscedasticity were evaluated using QQ plots and residual-versus-fitted plots. Count response variables were analyzed using GLMMs: a negative binomial distribution was used for the number of vibrational pulses, whereas a Poisson distribution was used for the number of flowers visited after departure from the focal treatment flower using the “glmer” and “glmer.nb” function from the lme4 package (Bates et al. 2015). The adequacy of the GLMMs was evaluated using simulation-based residual diagnostics implemented in the DHARMA package (Hartig 2026), including tests of overdispersion, uniformity, outliers, and zero inflation. These diagnostics provided no evidence of model violations. Treatment effects were evaluated globally to determine whether responses differed among pollen-availability levels, using F-tests with Satterthwaite’s approximation for LMMs using the “anova” function from the lmerTest package (Kuznetsova et al. 2017) and Wald χ^2 tests for GLMMs using the “Anova” function from the car package (Fox and Weisberg 2019). Treatment contrasts were examined using the estimated coefficients and associated test statistics obtained from the model summaries using the “summary” function from the lme4 package (Bates et al. 2015). Because the 100% pollen-availability treatment (the unmanipulated control) was set as the reference level, each coefficient represented the difference between a given pollen-restriction treatment and the control.

Additionally, we applied principal component analysis (PCA) to the descriptors of bee behavior in flowers (handling time, buzzing time, grooming time, number of vibrational pulses, peak frequency, and relative amplitude peak), following the recommendations of Björklund (2019). Randomization tests were used to identify which principal components were informative and which loadings were associated with each component. As the PCA results were consistent with the individual analyses of each behavioral variable, we chose to present these data only in Figures S3–S7. All graphical outputs were generated using the ggplot2 package (Wickham 2016). All statistical analyses were performed in R software (version 4.6.0; R Core Team 2026), using the RStudio interface (version 4.6.0; R Core Team 2026).

3 | Results

3.1 | Is the Visual Attractiveness of the Two-Stamen Morphs Different for *B. morio* Bees?

The frequency of *B. morio* visits to flowers was affected only by the complete removal of poricidal anthers (RT), which reduced the number of visits relative to the control ($\beta = -0.75 \pm 0.25$, $z = -3.01$, and $p = 0.003$; Table S2; Figure 2A). None of the other stamen-removal treatments differed from the control. Similarly, visit duration was shorter only when all stamens were removed ($\beta = -0.91 \pm 0.41$, $t = -2.25$, and $p = 0.029$; Table S2; Figure 2B), with no differences detected for the remaining stamen-removal conditions.

3.2 | Does Experimentally Restricted Pollen Availability Influence Bee Foraging Behavior on Flowers?

In the experiment testing the effects of pollen restriction on bee behavior, we recorded 62 visits of *B. morio* to different flowers: 16 visits to flowers with 100% pollen availability (control), 16 to flowers with 64%, 15 to flowers with 36%, and 15 to flowers with complete obstruction of the anther pores (0%).

Gradual pollen restriction did not affect most behavioral descriptors of *B. morio* (Table 1), including buzzing time (Figure 3B), the number of vibrational pulses (Figure 3D), the fundamental frequency of vibrations (Figure 3E), and the relative peak amplitude (Figure 3F). Behavioral differences relative to the control were detected only under complete pollen obstruction for handling and grooming times (Table 1; Figure 3A,C). Bees spent less time handling flowers with completely obstructed anthers ($\beta = -0.61 \pm 0.27$, $t = -2.26$, and $p = 0.028$; Table 1; Figure 3A), corresponding to an average reduction of approximately 10 s (47%). Likewise, grooming time was markedly reduced in flowers with complete anther obstruction ($\beta = -1.60 \pm 0.35$, $t = -4.57$, $p < 0.001$; Table 1; Figure 3C), with bees grooming for only 0.8 ± 0.3 s on average, representing a

reduction of approximately 94% relative to the control treatment (14.5 s). In 12 of the 15 visits to flowers with complete obstruction, bees did not perform any grooming behavior despite repeatedly buzzing the anthers, as observed in the control treatment.

Finally, bees visited fewer subsequent flowers only after visiting experimental flowers with completely obstructed anther pores ($\beta = -2.22 \pm 1.06$, $z = -2.11$, $p < 0.035$, Table 1; Figure 4), with nearly all bees made no additional flower visits after encountering this treatment.

4 | Discussion

In this study, we investigated whether the flower-visit behavior of *B. morio* bees on *C. latistipula* is altered by experimentally restricting pollen release from the poricidal anthers. To test our hypotheses about the impact of the restriction of the floral resource, we first evaluated the assumption that bees visually identify the different anther morphs, smaller and larger (Figure 2A,B; Table S2). Since bees showed no visual discrimination between anther types, we manipulated pollen availability by physically obstructing the anther pores. Contrary to our hypothesis, bees did not adjust their buzz frequency or amplitude in response to these pollen release restrictions. We hypothesized that reduced pollen availability would lead to shorter handling and buzzing times, fewer vibrational pulses, and increased vibration amplitude. However, even under the extreme condition of total pore obstruction, we observed no change in these bee behavioral descriptors (Figure 3A–F). The only alteration was an almost complete absence of body grooming behavior of bees (Figure 3C), which fully accounted for the reduction in total handling time on flowers with maximal pollen restriction. Importantly, although within-flower vibrational behavior remained remarkably invariant, bees did respond at a broader spatial scale: As expected, after visiting flowers with total obstruction of anther pores, individuals reduced the number of subsequent visits within the population (Figure 4). Below,

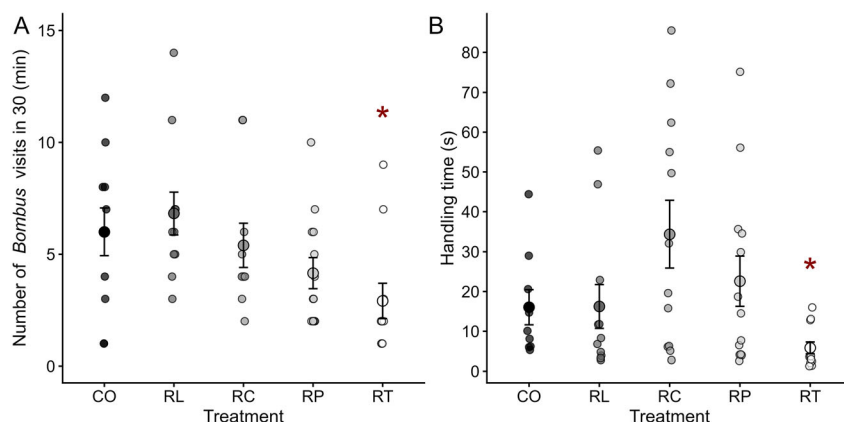


FIGURE 2 | Visitation and handling of *Bombus morio* bees in flowers of *Chamaecrista latistipula* under different anther removal treatments. Treatments were: CO, control (intact flowers); RL, removal of long anthers; RC, removal of short anthers; RP, partial anther removal (four short and three long anthers removed); and RT, total anther removal. (A) Mean number of visits per flower during 30-min observation periods and (B) mean handling time per visit. Centered circles indicate the means, and error bars represent $\pm$ standard error for each treatment. The red asterisks (*) identify treatments that differed from the control according to the corresponding mixed-model contrasts ($p < 0.05$).

TABLE 1 | Means $\pm$ standard deviations of behavioral descriptors recorded for *Bombus morio* bees visiting *Chamaecrista latistipula* flowers under different levels of pollen availability. The overall effect of pollen availability was evaluated using F-tests with Satterthwaite's approximation for denominator degrees of freedom for linear mixed models and Wald χ^2 tests for generalized linear mixed models. Treatment contrasts were examined to compare the control (100% pollen availability) with each reduced-pollen treatment, including the complete obstruction treatment (0% pollen availability). Plant identity was included as a random intercept in all models.

Behavior descriptors	Pollen availability (4 levels)				Statistics				
	100% available pollen (control)	64% of pollen available	36% of pollen available	0% of pollen available	Probability distribution	F or χ^2 statistics	N	P	Graphics
Handling time, s	20.7 $\pm$ 16.1	24.7 $\pm$ 22.1	20.3 $\pm$ 14.8	10.9 $\pm$ 8.3*	Gaussian	3.2	62	0.03	Figure 3A
Buzzing time, s	7.8 $\pm$ 3.1	11.3 $\pm$ 7.3	8.9 $\pm$ 4.7	8.3 $\pm$ 5.6	Gaussian	1.1	62	0.35	Figure 3B
Grooming time, s	14.5 $\pm$ 15.1	10.3 $\pm$ 15.9	18.7 $\pm$ 18.3	0.8 $\pm$ 0.3*	Gaussian	12.6	62	<0.001	Figure 3C
Number of vibration pulses	8.1 $\pm$ 4.0	10.4 $\pm$ 4.8	8.5 $\pm$ 3.7	8.1 $\pm$ 6.0	Negative Binomial	1.8	62	0.61	Figure 3D
Fundamental frequency, HZ	241.7 $\pm$ 30.1	262.4 $\pm$ 39.4	259.1 $\pm$ 41.5	248.7 $\pm$ 36.9	Gaussian	0.9	62	0.44	Figure 3E
Peak relative amplitude, dB	-25.6 $\pm$ 5.8	-24.6 $\pm$ 6.5	-25 $\pm$ 6.1	-26.9 $\pm$ 5.9	Gaussian	1.0	62	0.41	Figure 3F
Subsequent visits after the focal flower	0.7 $\pm$ 1.2	0.8 $\pm$ 1.0	0.7 $\pm$ 1.1	0.07 $\pm$ 0.25*	Poisson	5.2	62	0.16	Figure 4

Note: Asterisks (*) indicate pollen availability levels that differed from the control in the mixed-model contrasts ($p < 0.05$).

we discuss potential mechanisms that could explain this notable invariance in vibrational behavior of *B. morio* on flowers of *C. latistipula*.

The observed discrepancy between the flexible foraging behavior reported for pollinators in other systems (Buchmann and Cane 1989; Harder 1990; Russell et al. 2016; Knauer et al. 2021) and the invariant bee behavior on *C. latistipula* flowers may stem from the absence of prior visitor cues on our experimental flowers. In natural conditions, Bombini bees rely strongly on chemical cues (scent marks) left by themselves or by other visitors to assess a flower's profitability and avoid recently depleted flowers (Goulson et al. 1998; Pearce et al. 2017; Leadbeater and Chittka 2011). In our experiment, however, we used virgin flowers, never previously visited, and ensured that each was visited only once. This design effectively removed these chemical cues from the environment. Because all experimental anthers remained full of pollen but were differently blocked, bees may not have been able to detect actual differences in pollen accessibility before attempting extraction. Therefore, rather than revealing the absence of sensory mechanisms to detect residual pollen, our results likely reflect a scenario in which *B. morio* had no reliable cues, chemical, visual, or mechanical, to assess pollen availability prior to contact. In this context, bees resorted to their default behavioral pattern, producing vigorous and prolonged vibrations, as an initial exploratory strategy. In addition to chemical cues, visual evidence of previous handling (e.g., characteristic anther damage from pollinator biting; Woodrow et al. 2024; Nogueira et al. 2018) was also absent. This specialization is evident in the bee's ability to grasp all ten stamens simultaneously, a behavior that facilitates the transmission of vibrations

through direct contact between the bee's ventral surface and the anther cone (Barbosa et al. 2023; Woodrow et al. 2024). Thus, the lack of both chemical and visual marks may have caused bees to treat all experimental flowers as unvisited, resulting in the consistent and invariable use of their most efficient vibrational behavior, regardless of the actual pollen availability. Additionally, forager bees are capable of learning and refining floral manipulation through repeated visits (Russell et al. 2016; Takagi and Ohashi 2025). Because *B. morio* frequently visits *C. latistipula* and is highly specialized in exploiting its poricidal anthers, experienced individuals may rely on a previously learned and efficient buzzing routine rather than adjusting their behavior during each visit. This learnt motor pattern may further reinforce the stereotyped vibrational behavior observed in our experiment.

Beyond the reliance on external cues, the innate and energetically costly nature of buzz pollination provides a compelling explanation for the observed behavioral invariance. The biomechanical constraints involved in transmitting vibrations to poricidal anthers may limit the degree to which bees can fine-tune their buzzing behavior during individual visits (Pankratz and Russell 2026). Unlike systems where bees adjust vibrations based on resource cues (Buchmann and Cane 1989; Harder 1990), we propose that the high efficiency of the interaction between *B. morio* and *C. latistipula* renders such plasticity unnecessary. As the primary pollinator of this plant (Nogueira et al. 2018; Barbosa et al. 2023), *B. morio* is morphologically specialized for its floral architecture. This specialization is evident in the bee's ability to grasp all 10 stamens simultaneously, suggesting an optimized and stereotypical foraging

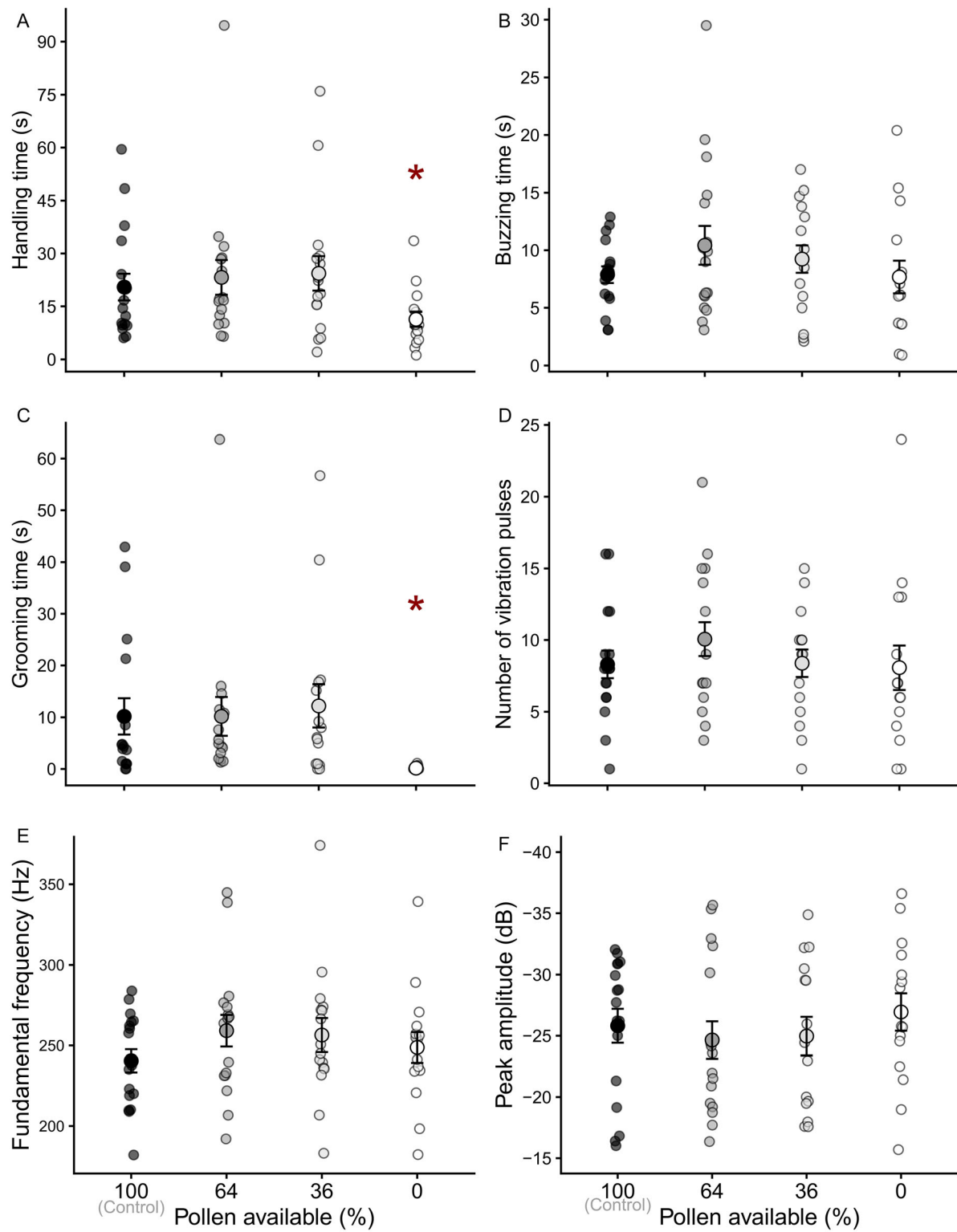


FIGURE 3 | Effects of the percentage of pollen available for extraction from *Chamaecrista latistipula* flowers on the behavior of *Bombus morio*: handling time (A), buzzing time (B), grooming time (C), number of vibrational pulses (D), fundamental frequency of buzzes (E), and relative peak sound amplitude (F). Centered circles and error bars indicate the mean $\pm$ standard error at each pollen availability level. The red asterisks (*) indicate treatments that differed from the control (100% pollen availability) in the corresponding mixed-model contrasts ($p < 0.05$).

routine. This morphological fit allows this bee species to extract substantial and predictable quantities of pollen with each visit ($98\% \pm 0.02\%$; Barbosa et al. 2023). This efficiency in pollen extraction is supported by an exceptionally high handling time (20.7 ± 16.1 s in flowers with 100% pollen), considerably longer than other buzz-pollinating bees like *Ptiloglossa arizonensis*

In this species, Souza et al. (2024) showed that rhizobial association increases floral attractiveness, indicating that the plant may allocate resources consistently to floral traits. Moreover, Fabaceae pollen generally contains relatively high protein levels (20%–40% crude protein; Roulston et al. 2000), values that are above those reported for several other families, such as Asteraceae (e.g., *Taraxacum officinale*, ~13%; Roulston et al. 2000). Although our study did not quantify pollen composition and quality, investigating how nitrogen-driven variation in pollen quality shape vibration intensity may help clarify why *B. morio* maintains consistent buzzing behavior across flowers.

From the perspective of floral fitness, the behavioral invariability of *B. morio* can confer reproductive advantages on *C. latistipula*. The consistent application of high-amplitude vibrations ensures that even flowers with low pollen production undergo efficient vibrations, potentially maximizing the rate of viable pollen grain removal and export. This behavioral pattern eliminates the risk of aborted visits or low pollinator efficacy in suboptimal flowers, ensuring a continuous flow of pollen between flowers and individuals in the population. Furthermore, the reduction in handling time observed in flowers with poricidal anther completely blocked, resulting from the absence of grooming behavior, induces a higher turnover of visited flowers per unit of time. This can increase the overall visitation rate on the plant as the pollinator allocates less time to depleted sources and more time to searching for new flowers. Thus, the stereotyped behavior of the pollinator, possibly shaped by the high average quality of the resource (e.g., Souza et al. 2024; Dollard et al. 2025), promotes a more reliable and efficient pollination service for *C. latistipula*, favoring the persistence and robustness of this mutualism in the face of intrinsic variation in pollen supply among flowers.

Our results challenge the traditional expectation derived predominantly from Northern Hemisphere systems that pollinators should exhibit fine behavioral plasticity and adjustments in response to intrafloral resource variation. This perspective is consistent with recent evidence suggesting that buzz pollination systems may not be as evolutionarily stable or uniform as previously assumed (Nery et al. 2024). This study demonstrates that average behavioral constancy, rather than flexibility, may represent the optimal foraging strategy in the highly specialized mutualism between *B. morio* and *C. latistipula* in the Brazilian Cerrado. Importantly, this behavioral constancy does not imply an absence of adaptive responses but rather a shift in the scale at which plasticity is expressed: while vibrational behavior remains stereotyped within flowers, flexibility emerges in postvisit decisions at the population level, particularly following nonrewarding experiences. We propose that this pattern is supported by a network of interconnected factors: reliance on previous visitor cues (chemical and visual) to assess resources, the costly and innate nature of vibration, resource evaluation at the floral patch scale, and probably the high quality and predictability of the resource, potentially mediated by multitrophic interactions with nitrogen-fixing bacteria (Souza et al. 2024; Dollard et al. 2025). Our findings highlight that examples of the Global South mutualisms, often embedded in complex ecological networks and historically less studied, may operate under distinct ecological rules. Therefore, this work not only advances our

understanding of buzz pollination but also shows the importance of Global South studies for enriching general ecological models, revealing how tropical biodiversity challenges and enriches our theoretical understanding of plant–animal interactions.

5 | Conclusion

Contrary to expectations rooted in classical optimal foraging theory, our study shows that the vibrational behavior of the bee *B. morio* remains remarkably invariable across an experimental gradient of pollen restriction in *C. latistipula* flowers. This invariability persisted even under the extreme condition of total obstruction of anther pores and extended to the biomechanical properties of vibrations. The only adjustable responses were reductions in handling time and grooming time, an energy-saving mechanism associated with the absence of pollen removal, and a reduction in patch residence following visits to flowers with complete obstruction, reflecting behavioral flexibility at the population scale rather than within flowers. These results indicate that, in this Cerrado mutualistic system, the optimal strategy is not fine behavioral plasticity but the consistent deployment of an innate, highly efficient, stereotyped behavior. We propose that this pattern emerges from a combination of factors: (1) reliance on previous-visitor cues (chemical and visual), absent in our experimental design, as a primary mechanism for resource assessment; (2) the high energetic cost of vibration, which favors a standard high-efficiency strategy; (3) profitability being evaluated at the patch scale, where suboptimal handling of a single flower incurs negligible costs; and fundamentally, and (4) the high quality and reliability of the pollen resource, potentially reinforced by multitrophic interactions with nitrogen-fixing bacteria, making substantial behavioral adjustments disadvantageous. Looking ahead, our findings highlight the need to (1) incorporate simulations of previous-visitor signals into experimental designs and (2) investigate how multitrophic interactions (e.g., soil–plant–pollinator) shape animal behavior and pollination dynamics. These research directions are essential for developing an ecological theory that encompasses the global complexity of pollination mutualisms.

Acknowledgments

We are grateful to all members of the Plant–Animal Interactions Laboratory (LIPA) at the Federal University of ABC (UFABC), especially to Caian S. Gerolamo for assistance with the PCA. We also thank Professors Laura Leal and Marcelo Rossi for their valuable suggestions during the undergraduate thesis stage. We acknowledge the support of the Multiuser Center for Biodiversity and Conservation (CMBC-PROPE/UFABC) for providing facilities for plant cultivation, experimental work and equipment's (FAPESP grant no. 2021/09239-2). This study was funded by an undergraduate research fellowship awarded to G.V.M. through the Scientific Initiation Program (PIC) of UFABC (Call no. 01/2020, institutionally funded), and by a Master's fellowship from FAPESP (grant no. 2024/02825-1), which supported the preparation of this manuscript. B.C.B. acknowledges FAPESP for a PhD fellowship (grant no. 2023/00065-7) and for the scientific initiation scholarship (grant no. 2020/09090-6). A.N. acknowledges the São Paulo Research Foundation (FAPESP, grant no. 2019/19544-7) for a Young Investigator fellowship, and the National Council for Scientific and

Technological Development (CNPq) for a Productivity in Research fellowship – Level 2. Generative artificial intelligence (ChatGPT, OpenAI) was used to assist with language editing, improving grammar, clarity, and the overall organization of the manuscript. The authors critically reviewed and edited all AI-assisted text and take full responsibility for the final content. No AI tools were used for study design, data collection, data analysis, interpretation of results, or the generation of scientific conclusions.

The Article Processing Charge for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier: 00x0ma614).

Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (Grants 19/19544-7, 21/09239-2, 20/09090-6, 23/00065-7, 24/02825-1), Conselho Nacional de Desenvolvimento Científico e Tecnológico (Grant 312389/2023-0), and Universidade Federal do ABC (Grant 14203 (Edital 01/2020 – UFABC)).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data and R scripts supporting the results of this study are available in Figshare: <https://doi.org/10.6084/m9.figshare.32680266>.

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- three experimental treatments: CO (control), CP (petals glued), and CA (anthers glued). Small dots represent individual censuses ($n = 30$ flowers), while larger filled circles with error bars show the mean $\pm$ SE for each treatment. The experiment aimed to test whether the use of glue on floral structures would alter pollinator visitation. A generalized linear mixed model with Poisson error distribution (plant identity as a random effect) indicated no significant effect of treatment on visitation rates (CO vs. CP: $z = -1.23$, $p = 0.22$; CO vs. CA: $z = -1.58$, $p = 0.11$). The dispersion parameter was < 1 (0.54), indicating no evidence of overdispersion in the model.
- Figure S3:** Randomization test of PCA eigenvalues used to identify which principal components capture biologically meaningful information from the original variables describing the behavior of *Bombus morio*. The boxplots represent the distribution of randomized eigenvalues (null distribution), while the red points indicate the observed eigenvalues obtained from the PCA. A significant departure of the observed eigenvalue from the null distribution suggests that the corresponding axis explains more structure than expected by chance. Only the first principal component (PC1) was retained as biologically relevant in this study ($p < 0.001$), highlighting its importance in summarizing patterns of behavioral variation.
- Figure S4:** Randomization test of the loadings contributing to the first principal component (PC1) of the PCA. The boxplots represent the distribution of randomized loadings (null distribution), whereas the red points indicate the observed loadings derived from the PCA. A significant deviation of the observed loadings from the null distribution identifies variables that contribute more strongly to PC1 than expected by chance. In this case, the observed loadings of vibration pulse number, handling time, buzzing time, and grooming time were found to be biologically relevant ($p < 0.001$), confirming their importance in shaping the behavioral patterns captured by PC1.
- Figure S5:** Principal component analysis (PCA) of the variables describing the foraging behavior of *Bombus morio* on flowers of *Chamaecrista latistipula*. The first two principal components (PC1 and PC2) explain 50% and 17.9% of the variance, respectively. Behavioral variables included peak frequency, peak relative amplitude, grooming time, buzzing time, handling time, and number of vibrations. Points in the plot are colored according to pollen availability, as manipulated through anther pore sealing. Arrows indicate the direction and strength of the relationship between each behavioral variable and the corresponding principal component.
- Figure S6:** Principal component analysis (PCA) of the significant behavioral variables of *Bombus morio* on *Chamaecrista latistipula* flowers, identified through a randomization test. The first principal component (PC1) explains 50% of the total variance and is primarily influenced by handling time (A), vibration duration (B), number of buzz pulses (C), and grooming time (D), in decreasing order of contribution. Higher PC1 scores are inversely related to the values of these behavioral variables, indicating that individuals with lower handling and vibration times tend to score higher on this axis. Each point in the plot represents an individual bee's behavior, and colors indicate the percentage of pollen available in the flowers, highlighting how resource availability may modulate foraging behavior. This analysis provides an integrated view of how multiple aspects of bee behavior are structured and correlated in response to floral conditions.
- Figure S7:** Relationship between the percentage of pollen available in *Chamaecrista latistipula* flowers and the foraging behavior of *Bombus morio*. The first principal component (PC1) represents the overall behavioral response of the bees, integrating multiple aspects such as handling time, vibration duration, number of buzz pulses, and cleaning time. PC1 did not differ significantly among treatments (ANOVA: $F = 2.64$; $p = 0.058$), indicating that general bee behavior was consistent regardless of experimental conditions. Each data point corresponds to an individual bee's behavior in relation to the pollen availability of the flower it visited. Black lines indicate the mean pollen percentage and standard deviation for each treatment. This figure allows visualization of how resource availability may influence bee foraging patterns, highlighting that despite differences in pollen levels, the overall behavioral strategy of *B. morio* remained relatively stable across flowers. Such analyses provide a comprehensive understanding of how multiple behavioral components interact and respond to ecological variables.
- Table S1:** Mean $\pm$ standard deviation of the amount of pollen present in the stamens of *C. latistipula*. Pollen quantity and pollen percentage were represented according to anther morph groups: the smaller morph composed of 7 stamens; the larger morph composed of 3 stamens; and all

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Experimental population of 30 individuals of *Chamaecrista latistipula* cultivated in pots and maintained in the experimental garden of the Federal University of ABC (UFABC), São Bernardo campus (23°40'40.8"S, 46°33'49.0"W). Plants were grown under natural environmental conditions, which allowed monitoring of flowering phenology, experimental stamen manipulations, and pollinator censuses during the study. **Figure S2:** Number of *Bombus* visits to *Chamaecrista latistipula* flowers recorded during 30-min censuses under

10 stamens combined. The total of 10 stamens corresponds to the sum of the short and long stamens, which were counted as separate groups. **Table S2:** Means $\pm$ standard deviations of behavioral descriptors of *Bombus morio* in different scenarios of stamen manipulation in flowers of *Chamaecrista latistipula*, recorded during 30-min censuses. The overall effect of stamen removal was evaluated using F-tests with Satterthwaite's approximation for denominator degrees of freedom for linear mixed models, and Wald χ^2 tests for generalized linear mixed models. The acronyms refer to the experimental conditions: CO, intact flower; RL, removal of long stamens; RC, removal of short stamens; RP, partial removal of stamens (removal of three long stamens and four short stamens); and RT, total removal of stamens. A logarithmic transformation was applied to the handling time (s) variable, as it did not meet model assumptions. Plant identity was included as a random intercept in all models. Asterisks (*) indicate values that differed from the control in the mixed-model contrasts ($p < 0.05$).