

Daily fluctuations in pollination effectiveness explain higher efficiency of native over exotic bees in *Lepechinia floribunda* (Lamiaceae)

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- **Background** Despite Stebbins' principle of the most efficient pollinator was proposed decades ago, most important pollinators are still mainly identified using the frequency of visits to flowers. This shortcoming faces us with a gap between the characterization of the flower visitors of a plant species and reliable estimation of the plant fitness consequences of the mutualistic interaction. The performance of a mutualistic visitor depends on its abundance, behaviour, its effectiveness (pollen removal and deposition per unit time), and efficiency (seed set per unit time) conditioned by the temporal matching between pollinators activity and temporal patterns of maturation of flowers sexual functions. Although recent attempts to provide a conceptual and methodological framework to characterize pollinators' performance, few have combined all key elements of visitors and plants to provide an accurate estimation of pollinators' performance under natural conditions.

- **Methods** we complement information on flower biology and mating system of the subshrub *Lepechinia floribunda* (Lamiaceae) to provide a daily quantitative estimation of performance (effectiveness and efficiency) of the more abundant pollinators: native bumblebees (*Bombus* spp.) and leafcutter bees (*Megachile* sp.), and the exotic honeybee (*Apis mellifera*).
- **Key Results** Unlike honeybees or leafcutter bees, native bumblebees matched the daily pattern of nectar production and stigma receptivity, and showed higher effectiveness and efficiency. Despite the overabundance of honeybees, visits occurred mainly when stigmas were not receptive, thus reducing the honeybees' overall performance.
- **Conclusions** Bumblebees appear as the most important pollinators and potential historical mediators of reproductive trait evolution in *L. floribunda*. Because the production of seeds by bumblebees involved fewer pollen grains for plants and less investment in floral display than honeybees, contemporary and expected changes in pollinators abundance may affect future *L. floribunda* floral evolution. If bumblebees were to be further displaced by anthropogenic disturbance or by competition with honeybees, their lower efficiency will select for larger floral display increasing reproductive costs. This scenario may also impose selection to reduce dichogamy to match honeybee foraging activity.

Key words: foraging behavior, *Lepechinia floribunda*, outcrossing rate, plant–pollinator interactions, pollen deposition, pollen removal, Stebbins’ principle, visitation frequency.

INTRODUCTION

The relationship between flowering plants and pollinators seldom occurs in a pairwise fashion because the majority of plant species are visited by more than one pollinator species (Ashworth *et al.*, 2015). Thus, a central aspect is the identification of the floral visitors that exert the strongest positive effect on plant fitness (Mayer *et al.*, 2011). Following Stebbins principle of the most efficient pollinator (Stebbins, 1970), these are expected to impose selection on floral traits and will be considered key agents of selection to explain phenotypic evolution (Poblete Palacios *et al.*, 2019). However, even though the importance of a pollinator depends on its effect on plant fitness (Fenster *et al.*, 2004), there has been a historical bias to infer the ecological and evolutionary consequences of a plant-pollinator interaction based on its frequency of occurrence (Vazquez *et al.*, 2005). Although the frequency of visits to flowers provides a useful approximation, it is by no means a definitive assessment of pollinator performance and of its effect on plant fitness (Waser *et al.*, 1996). Despite that recent theoretical and methodological proposals argue for a more precise estimation of pollinators performance (Ne'eman *et al.*, 2010; Freitas, 2013; Schupp *et al.*, 2017, Minnaar *et al.*, 2019) the identification of the pollen vectors that play a central role in plant reproduction and evolution remains a major challenge and awaits further investigation.

Pollinator performance can be defined as the absolute contribution of a given pollen vector to plant fitness and involves at least two main sequential factors: (1) abundance of pollinators, and (2) pollen removal and deposition. The interaction between these two ultimately determines the role of each pollinator as a vector of gametes affecting mating and plant fitness (Herrera, 1987). The association between the abundance of pollinators and pollen removal/deposition performance is not necessarily linear, because less abundant pollinators may deposit more pollen per visit, and elicit a higher seed set than the most abundant ones (Zych, 2007; Barrios *et al.*, 2016), and vice versa (Sahli and Conner, 2007; Medel *et al.*, 2018).

Ne'eman *et al.* (2010) defined pollinator performance as resulting from pollination effectiveness and efficiency. Pollination effectiveness quantifies the ability of a floral visitor to remove and deposit pollen on stigmas per visit per unit time. Pollination efficiency indicates to what extent pollen deposition contributes to female plant fitness per visit per unit time (*i.e.* including pollen quality). These definitions reveal that an effective floral visitor in terms of pollen deposition could not be efficient in terms of seed production (Ne'eman *et al.*, 2010). This may occur in dichogamous species if pollinators activity is concentrated during only during one phase of sexual maturation, either when anthers open or stigmas are receptive (Zych, 2007). However, an efficient pollen vector is certainly effective.

The relationship between floral traits and interaction traits accounting for the variation in pollinators' performance in natural conditions is depicted in Figure 1. Availability of pollinators for a focal plant species in a given patch and community context usually depends on its floral display (Proctor *et al.*, 1996). Plants with large floral displays usually receive more visits (Harder and Johnson, 2009), and those with high visitation frequency produce more seeds. Nevertheless, these sequential events can be modulated by the flower/pollinator adjustments during each pollination event (Poblete Palacios *et al.*, 2019). From arrival to a flower, morphological matching between a pollinator and floral architecture, together with pollinator behavior, modulate handling time and effectiveness of both pollen removal and deposition (Barrios *et al.*, 2016). The efficiency of a pollinator is given by its contribution to the final fitness of a plant (Fig. 1). Thus, both pollination effectiveness and efficiency translate in the final performance of each pollinator species. In addition, within a population, pollen movement among plants and the resulting outcrossing rate depend on the coordination between the period of pollinator activity and the timing of maturation of sexual phases among flowers (Herrera, 1990). Hence, the variation in the number of receptive flowers along the day, relative to the timing and duration of

pollinator activity should also be taken into account for a reliable estimation of pollinator performance (Albercht *et al.*, 2012). Because daily fluctuations in environmental conditions and pollinators activity affect pollination effectiveness and efficiency (Fig. 1; Herrera, 1987, 1990), those pollinators that better match the population pattern of anther maturation and stigma receptivity will have greater performance.

Due to the multifactorial context of the pollination process, there is an important gap between the characterization of the floral visitor community in a given plant population and the quantitative assessment of pollinator performances. This gap limits our ability to identify the most important selective agents affecting floral evolution and manipulate efficient pollinators to warrant outcrossing in the context of population conservation and crop production.

The present study attempts to narrow this gap using as study system *Lepechinia floribunda* (Benth.) Epling (Lamiaceae), that is visited by several species of native bees, bumblebees, and the cosmopolitan honeybee *Apis mellifera* (Roldan and Ashworth, 2018). As native bees have a longer history of interaction with the selected plant species than honeybees, it is expected that native bees will show greater effectiveness and/or efficiency than honeybees. Nevertheless, if honey bees are more abundant than native bees (Magrach *et al.*, 2017; Valido *et al.*, 2019), a lower effectiveness and efficiency may be compensated with a higher visitation frequency. In the present study, we (1) described the floral biology of *L. floribunda* (daily patterns of both nectar secretion and stigma receptivity), (2) examined the relevance of pollinators for seed production through outcrossing rate (t) estimation; (3) assessed relative abundance, visitation rate, and relative visits of the whole community of floral visitors; and (4) compared pollination effectiveness (*i.e.* contribution of each pollinator to pollen removal and deposition per unit time) and efficiency (*i.e.* female fitness per pollinator per time unit) between native and non-native pollinators.

MATERIALS AND METHODS

System and study site

Lepechinia floribunda (Lamiaceae) is a perennial subshrub native of the montane forests in Argentina, Bolivia, and Peru commonly found in dry open habitats from 500 to 3500 meters above sea level (Epling, 1938). It is a dominant species that blooms from early October to late February, producing several short bilabiate white hermaphroditic flowers per plant throughout the flowering season. Receptive flowers last one day, present bifid stigmas and four ovules. They are incomplete protandrous (anthers mature before stigmas), and can self-fertilize autonomously (Roldan and Ashworth, 2018). Fruits mature approximately three weeks after pollination (Camina et al., 2018).

The study was performed in a natural population at the Reserva Natural Los Manantiales (31°9'40.34" S, 64°21'03.67" W) in Central Argentina where 164 tagged individuals were studied during three blooming seasons (2013-2014, 2014-2015, and 2018-2019).

Floral biology: nectar production, stigma receptivity, and mating system

To characterize the floral biology of *L. floribunda*, during the flowering season of 2014-2015, daily nectar production was calculated using 115 randomly selected floral buds from 26 plants bagged before 7:00 h. Opened flowers were harvested every hour (12-21 bagged flowers per hour) from 8:00 h to 15:00 h when nectar production dropped. Nectar volume (μl) and concentration ($\mu\text{g}/\mu\text{l}$) were recorded using 1 or 5 μl micro-caps and a temperature compensated hand refractometer (range concentration 0–32° BRIX units; American Optical 10431). These variables were used to calculate nectar sugar content (μg) following Kearns and Inouye (1993).

The percentage of receptive stigmas per hour was recorded using 253 randomly selected floral buds from 52 plants (15-68 bagged flowers per census) to estimate the population stigmatic receptivity throughout the day. After a preliminary evaluation, only pollen deposition on stigmatic branches opened at 90° produced seeds, thus only completely opened stigmas were considered receptive (Camina, 2018).

To characterize the mating system after the reproductive season of 2014-2015, we sowed 10–20 seeds for each of 15 plants in germination chambers following the protocol proposed by Ashworth *et al.* (2017) and, after one month, the first leaves were collected and freeze dried (n = 188 seedlings). We extracted DNA and amplified 12 microsatellites specifically developed for *L. floribunda* (molecular techniques details and microsat features are provided in Supplementary data Information S1).

We used a maximum likelihood approach to estimate the outcrossing rate by genotyping each family and the whole sample and using a mixed mating system model of Ritland and Jain (1981) implemented in the program MLTR v.3.4 (Ritland, 2002). MLTR calculates multilocus (t_m) and single-locus (t_s) outcrossing rates using the Newton–Raphson iteration. These estimations range between 0 (indicating complete selfing) and 1 (indicating complete outcrossing). Outcrossing rate standard errors and 95% confidence intervals were obtained from 1000 bootstrap replicates. The outcrossing rate tuning parameter, allele frequency tuning parameter and initial population outcrossing rate were set to 0.05, 0.1 and 0.5, respectively.

Floral visitor abundance

To identify the most abundant floral visitors we characterized the floral visitor community, its period of greatest activity and determined the abundance of its members by recording mean visitation rate (visits per minute (min); V_r), relative visits (visits per observed flowers; R_v), and relative abundance (R_a ; Table 1) of each floral visitors species during two consecutive reproductive seasons. Direct observations of visitors were made between 8.00 h and 17.00 h, in periods of 15 minutes per plant, during 10 days accumulating a total of 17 and 28 man-hours observation in the reproductive seasons of 2013-2014 and 2014-2015, respectively.

Based on this characterization of the floral visitor community, we carried out a series of experiments to determine pollination effectiveness and efficiency of the two most abundant visitors using individual performance measures (such as visitation frequency and pollen deposition on the stigma) and combining these individual measures following the approach proposed by Ne'eman et al. (2010; Table 1). To define the most important flower visitors, we considered their visitation consistency throughout two reproductive seasons (see results, and Table 2).

Following our previous characterization, subsequent estimations of pollinator performance were obtained for the honeybee (*Apis mellifera*), leafcutter bees (*Megachile* sp.), and a set of two functionally equivalent native bumblebee species (see below). Together, these species were responsible for 70-85 % of total visits during two consecutive years (see Table 2). Because the two *Bombus* species recorded (*B. pauloensis*, *B. opifex*), showed similar patterns of floral manipulation and have similar size and morphological features, in the following experiments and analyses they were combined under the same functional category (*i.e.* *Bombus* spp.).

Visitation frequency and handling time

During the flowering season of 2014-2015, visitation frequency (Vf) and handling time per visit (Ht) were recorded using a stopwatch for the three pollinator groups. Vf was calculated as the proportion of visited flowers per species per hour, and Ht as the time between arrival and departure from a flower (n = 190 observations; Table 1). Additionally, to explore the daily fluctuation in these variables we calculated Vf and Ht every hour for each species between 9:00 h and 13:00 h, the period of highest insect activity according to previous observations.

Pollen deposition and removal

During the flowering season of 2014-2015, pollen removal (Pr) and pollen deposition (Pd) by *A. mellifera*, *Megachile* sp. and *Bombus* spp. after one visit were calculated using a random sample of 54 focal plants. Two to six flowers (n = 193 total flowers) per plant were chosen early in the morning and bagged before anthesis to avoid possible early visits. Once stigmas became receptive and exposed their stigmatic lobules, one or two flowers per plant were used to estimate the total number of pollen grains before a pollinator visit (n = 65, these are considered control flowers). Anthers of selected flowers were harvested with a forceps to gently collect all its pollen and mounted it in a drop of stained glycerine jelly previously held on a slide (Baranzelli *et al.*, 2014). Remaining bagged flowers (n = 129) were exposed to the three pollinator groups. When stigmas became receptive one anther and the stigma of each flower were collected after one visit following the same procedure described above (one or two flowers per visitor per plant). The absolute number of pollen grains in the anthers and on the stigmas were counted using digital images from slides taken at 10x magnification with an Olympus DP71 camera attached to an Olympus SZX16 stereomicroscope. ImageJ free software (National Institute of Health, USA) was used to count the total number of pollen grains per anther and on the stigmatic lobules. For each visitor, Pr was estimated for each focal plant as the difference in

the number of pollen grains per anther, between non visited and visited flowers ($Pr_{\text{unvisited flowers}} - Pr_{\text{visited flowers}}$). Similarly, Pd was estimated as the difference in number of pollen grains on stigmatic branches between non visited and visited flowers (as control of flower manipulation; Table 1). These calculations were performed for every one hour interval between 9:00 h and 13:00 h to record daily fluctuations in pollen removal and deposition by each pollinator.

Fruit set and seed set

During the flowering season of 2018-2019, fruit/flower (Fs) and seed/fruit (Ss) were calculated for 33 focal plants after a single visit of either *A. mellifera* or *Bombus* spp. Although *Megachile* sp. was quite good at removing pollen (see Results), it presents very low effectiveness, visitation frequency and was almost absent during the flowering season when efficiency was estimated, thus it was not included in the final performance estimations. A total of 3-17 flowers per plant ($n = 256$ flowers) were bagged early in the morning before anthesis to avoid pollinator visits. Once stigmas became receptive, flowers were unbagged, exposed to visitation, monitored continuously and bagged again after the first visit. Four weeks later, ripe fruits were collected to obtain fruit and seed set per pollinator. Fs was estimated as the proportion of fruits per flower per plant, and Ss was calculated as the number of mature seeds per fruit (Table 1).

Pollination effectiveness and efficiency

Given that the proportion of receptive stigmas and nectar production in the population increases since flowers open in the morning until midday (see Results), pollination effectiveness was calculated for every one-hour interval during pollinator activity to obtain the daily variation pattern and more accurate estimation of the response variable. Following Ne'eman *et al.* (2010)

framework, pollination effectiveness per time unit (D_t) for both pollinators was estimated as the product between its visitation frequency (V_f) and the number of pollen grains removed from anthers (P_r) or deposited on the stigmas (P_d), as indicated below:

$$\text{Pollen exportation effectiveness: } D_{te} = V_f \times P_r \quad (1)$$

$$\text{Pollen deposition effectiveness: } D_{td} = V_f \times P_d \quad (2)$$

Pollination efficiency per time unit (P_E) was calculated as the proportion of the maximum number of seeds per flower produced after one visit (as a measure of quality pollen; Ne'eman *et al.* 2010) multiplied by the visitation frequency. Because our studied species had a constant number of four ovules per flower, P_E was estimated as indicated below:

$$\text{Pollination efficiency: } P_E = (S_s/4) \times V_f \quad (3)$$

Following the suggestion of Ne'eman *et al.* (2010), we set the upper limit of P_E at 1. Hence, $P_E = 1$ corresponds to a pollinator that visited all focal flowers during the observation period and these produced maximum seeds per fruit.

Statistical analyses

Floral biology To compare nectar production throughout the day, linear models were fitted using time as an independent factor, and nectar volume and concentration as response variables. These analyses were implemented in the statistical software R v.3.3.2 (R Core Team, 2017), using the *lm()* function. Post hoc comparisons were performed using the Tukey test with the functions *glht* and *cld* (package: *multcomp*; Torsten *et al.*, 2008). To depict daily total sugar production patterns

throughout the day, non-parametric cubic splines were performed using the product between nectar volume and concentration per hour as the response variable and time of the day as an independent variable. For this analysis, we used *gam* function (package: *mgcv*; Wood, 2006) in R. Smoothing parameters were obtained by minimizing the generalized cross-validations cores (Wood, 2008), and Bayesian standard errors were obtained according to Wood (2006).

Pollinator performance To disentangle differences in pollination performance between the two main visitor categories, generalized linear mixed-effect models (GLMM; Zuur *et al.*, 2009) implemented in R were applied individually to each component of performance as described in the previous sections (Vf, Ht, Pd, Pr, Fs, Ss). Models included flower visitor (*A. mellifera*, *Megachile* sp. or *Bombus* spp.) as a fixed effect, and plants as a random effect within flower visitor. Significance of the fixed effects was estimated using restricted maximum-likelihood estimations and AIC comparisons of models. For Vf, Ht, Pd, Pr variables, models were performed using a Gaussian error distribution and the function *lmer* (package: *lme4*; Bates *et al.*, 2015), while Fs and Ss were compared using a binomial distribution of errors (family = binomial (logit)) with the function *glmer* (package: *lme4*).

Because pollen effectiveness (D_t) was recorded every hour during pollinators activity, weighted mean effectiveness was obtained for each pollinator as $\sum_{t=0}^{t=n} D_t$ where n is the period of observation following equation (1) and (2). The uncertainty of D_t and P_E per hour was calculated by bootstrapping 1000 times. Resampling was performed with the function *boot* (package: *boot*; Canty and Ripley, 2016) in R. Comparison of D_t between pollinators for both pollen exportation (D_{te}) and pollen deposited on the stigma (D_{td}) was performed using estimates of effectiveness for the whole period of pollinators activity (9:00 h - 13:00 h) and for the period when more than 70 % of the opened flowers had their stigma receptive in the population (11:00 h - 13:00 h; see Figure 2). Comparisons were performed using linear models using *lm()* function in R. In all

cases, we implemented post hoc multiple comparisons using Tukey range test with the functions *glht* and *cld*.

Comparison of P_E between *A. mellifera* and *Bombus* spp., following equation (3), was performed using Zero-Inflated Negative Binomial (ZINB) models to consider overdispersion and excess of zeros in the data. ZINB model was tested as suggested by Zuur *et al.* (2009; see Results), using the *zeroinfl* function (package: *pscl*; Zeileis *et al.*, 2008).

We established the significance level at a p-value <0.05 in all analyses. All response variables satisfied homoscedasticity and independence of error. Finally, we used *ggplot* function (package: *ggplot2*; Wickham, 2009) to build bar plots and *lineplotCI* function (package: *sciplot*; Murdoch, 2017) to build daily variation plots.

RESULTS

Floral biology

Early in the morning, mean (\pm s.d.) nectar volume was 2.4 ± 1.1 (μL), with a mean sugar concentration of 2.22 ± 0.70 ($\mu\text{g}/\mu\text{L}$). Nectar volume continuously increased throughout the day reaching 9.4 ± 1.6 μL before 15:00 pm. There were significant differences in nectar volume across time ($F = 3.794$; $p = 0.001$), in particular among the first hours of the morning (8:00 h and 9:00 h), midday (10:00 h to 14:00 h) and afternoon (15:00 h; Fig. 2A). Sugar concentration showed a nearly decreasing trend throughout the day, with a peak around 12:00 pm (2.5 ± 1.4 $\mu\text{g}/\mu\text{L}$), and a minimum concentration at 15:00 pm (0.8 ± 0.1 $\mu\text{g}/\mu\text{L}$; Fig. 2A) but without statistically significant differences across time ($F = 1.959$; $p = 0.073$). Throughout the day, flowers produced an average of 5.9 ± 0.4 μL nectar with 1.6 ± 0.1 $\mu\text{g}/\mu\text{L}$ sugar per flower. Sugar

production increased throughout the day (explained deviance= 79.7%; $p < 0.0001$), with a peak around 12:00 pm - 13:00 pm (9.4 ± 0.5 mg; Fig. 2B).

Proportion of receptive stigmas followed a similar trend as volume and sugar production. The first receptive stigmas were observed early in the morning around 8:00 h but in low frequency (20%). It was not until 11:00 h when more than 70% of the observed flowers had their stigmas open with an angle of more than 90° that indicated receptiveness. By 13:00 h more than 80% of stigmas were receptive (Fig. 2B).

Based on a random sample of 15 maternal plant families, genetic analyses indicated that their progenies ($n = 188$ seedlings) showed a total of 42 alleles using 12 nuclear microsatellite loci with a range of two to six alleles per locus. Multilocus outcrossing rate estimation (t_m) at family level ranged between 0.235 ± 0.06 and 1.000 ± 0.249 , (population mean \pm s.d. = 0.753 ± 0.113 ; details in Supplementary data Information S1).

Flower visitor assemblage

Our censuses of floral visitors during two seasons recorded a total of nine bee species from three families (Apidae, Halictidae, Megachilidae), and one hummingbird, *Chlorostilbon lucidus* (Trochilidae; Table 2). Hummingbirds were observed only in one season (in 2013-2014 season), while other visitors appeared in both seasons (Table 2). The sweat bee *Dialictus* sp., appeared at noon and afternoon. Taking all floral visitors into account, the highest V_r and R_v were recorded in the morning (0.32 visit/min; 0.13 visit/flower) and early afternoon (0.18 visit/min; 0.09 visit/flower). All floral visitors were observed either collecting pollen, nectar or both. Together, bumblebees, leafcutter bees, and honeybees represented more than 70% and 85% of the observed visits in 2013-2014 and 2014-2015 seasons, respectively (Table 2). Moreover,

these visitors appeared throughout the day with the highest visitation rates per flower and/or per minute (Table 2).

Visitation frequency and handling time

Average visitation frequency (Vf) was significantly higher for *A. mellifera* (1.29 ± 0.21 visits/flower/hour) than for *Bombus* spp. (0.83 ± 0.13 visits/flower/hour) or *Megachile* sp. (0.62 ± 0.08 visits/flower/hour; $F = 4.67$; $p = 0.0013$; Fig. 3A). *Apis mellifera* was the main visitor until 11:00 am, whereas *Bombus* spp. increased its frequency between 11:00 h and 13:00 h (Fig. 3B). *Megachile* sp. showed a low but constant Vf throughout the day. Handling time (Ht) varied between flower visitors ($F = 5.85$; $p = 0.005$). Post Hoc comparisons showed that *Bombus* spp. had, on average, significantly shorter handling time (2.30 ± 1.72 s/flower) than *Megachile* sp. (5.56 ± 3.53 s/flower) and *A. mellifera* (7.09 ± 3.70 s/flower); Fig. 3C). While *A. mellifera* and *Megachile* sp. showed a consistent reduction throughout the day in the handling time per flower, *Bombus* spp. was much more constant in this regard (Fig. 3D). Despite differences detected between pollinators, a significant plant effect on handling time per visit was also detected ($\Delta AIC = 3,130$; $p = 0.007$), accounting for 10 % of the total variation.

Pollen removal and deposition

The number of pollen grains available per anther of non-visited flowers ranged between 1301 and 2860, and the number of pollen grains removed per visitor species ranged between 0 and 2529 (mean \pm s.d = 968 ± 514). The estimated percentage of pollen removed per visit ranged from 0 to 94.47%. Pollen removal (Pr) was significantly different between pollinators ($F = 37.847$; $p < 0.001$). *Megachile* sp. removed around 20% more pollen per visit than *Bombus* sp. and 50% more than *A. mellifera* (Fig. 3E). The three species showed relatively constant removal

patterns throughout the day (Fig. 3F). Pollen grains deposited on the stigmas (Pd) varied between 0 and 80 grains per flower (mean \pm s.d = 5.84 ± 13.09) and showed a rather constant pattern throughout the day. No significant differences were detected among visitors ($F = 0.896$; $p = 0.350$; Fig. 3G, H). Variation among plants also accounted for a significant amount of variation in Pr ($\Delta AIC = 9.478$; $p = 0.002$), explaining 29 % of the variation.

Fruit set and seed set

Among tagged plants, fruit/flower (Fs) varied from 33% to 100%, and seed/fruit (Ss) from 0% to 100%. There were significant differences in Fs and Ss between flower visitors ($z = 5.891$, $p < 0.001$). On a single visit basis, *Bombus* spp. had both Fs and Ss about 40% higher than *A. mellifera* (Fig. 4).

Pollinator effectiveness and efficiency

The comparison of pollination effectiveness between floral visitors (D_t) throughout the whole observation period (9:00 h - 13:00 h), revealed similar levels of pollen removal effectiveness (D_{te}) across time intervals ($F = 79.507$; $p = 0.710$, Fig. 5A). In turn, significant differences in pollen deposition effectiveness (D_{td}) between *A. mellifera* and *Bombus* spp. respect to *Megachile* sp. were detected ($F = 3.398$; $p = 0.043$; Fig. 5A). Nevertheless, at the time of the day when more than 70% of stigmas were receptive (between 11:00 h and 13:00 h), the effectiveness of *Bombus* spp. removing ($D_{te} = 1186.5 \pm 20$) and depositing ($D_{td} = 6 \pm 0.25$) pollen grains was significantly higher than that of *A. mellifera* ($D_{te} = 496 \pm 12$; $D_{td} = 3 \pm 0.25$) and *Megachile* sp. ($D_{te} = 1024 \pm 20$; $D_{td} = 1 \pm 0.03$; D_{te} : $F = 417.58$, $p < 0.001$; D_{td} : $F = 156.7$, $p = 0.050$; Fig. 5). During this period, the ratio between pollen grains removed and deposited per visit was higher for *Bombus* spp. than for *A. mellifera* and *Megachile* sp. *Bombus* spp. had its

highest effectiveness early in the morning and after midday, *A. mellifera* showed a peak of high effectiveness at 10:00 h, while *Megachile* sp. showed a low but constant pattern of effectiveness (Fig. 5).

Given the low level of effectiveness and visitation frequency of *Megachile* sp. as well as its absence during one of the flowering seasons, it was not possible to obtain enough replicates to calculate pollination efficiency (PE) for this floral visitor. Pollination efficiency (P_E) of *Bombus* spp. per hour was significantly higher than that of *A. mellifera* (0.38 ± 0.01 versus 0.14 ± 0.01) ($\beta = -2.17$, $SE = 0.12$, $z = 17.59$, $p < 0.001$; Fig. 6A). Taking into account all the flowers observed in the population, this means that the per hour contribution to maximal seed set per visited flower was 2.7 times higher for *Bombus* spp. despite it being a less abundant pollinator. In other words, *Bombus* spp. needed less than three visits to reach maximum seed set whereas *A. mellifera* needed more than six to reach that maximum. This was due to the lower probability of setting seeds after one visit by a honeybee than by a bumblebee. (Fig. 6B).

DISCUSSION

We were able to disentangle pollinator performance of honeybees (*A. mellifera*), bumblebees (*Bombus* spp.) and leafcutter bees (*Megachile* sp.) through quantitative estimation of effectiveness and efficiency (*sensu* Ne'eman *et al.*, 2010), despite these pollinator groups belong to the same functional guild. As expected, honeybees were much more abundant on *L. floribunda* flowers than native bees. However, only one of the native visitors (*Bombus* spp.) was significantly more efficient than exotic bees due to its stronger overlap with daily phenological patterns of stigma receptivity and nectar production. This temporal matching accounted for the higher effectiveness and efficiency of bumblebees over honeybees and leafcutter bees. Results demonstrate that the higher visitation frequency of honeybees was not enough to surpass the

higher effectiveness and efficiency of the native bumblebees. Thus, bumblebees are likely the most important pollinators affecting the evolution of reproductive traits in *L. floribunda* through their positive effect on plant fitness. Under present levels of honeybees' performance, plants should produce three times more flowers, or honeybees should duplicate their visitation frequency, to attain a seed production per hour as high as it is attained by the native bumblebees. This scenario may impose a strong selection favouring an increase in floral display and a reduction in dichogamy to match honeybees foraging activity during the day if bumblebees were to be displaced by anthropogenic disturbance or by competition with honeybees. Thus, replacement of functions are likely within the pollinator assemblage of *L. floribunda*, but it would not be free of reproductive costs (Ashworth *et al.* 2015).

Over the years, several proxies for pollinator performance have been used, but a strong bias toward using the visitation frequency still persists (Dafni *et al.*, 2005; Willmer *et al.*, 2017). Estimations include the percent of open flowers with signals that pollinators touched the stigma, amount of pollen removal or deposition, or handling time, among others (Ne'eman *et al.*, 2010). However, few studies have combined any of these estimates with final fitness consequence of the pollination event (*i.e.* pollination efficiency; see Barrios *et al.*, 2016; Ma *et al.*, 2018, Valverde *et al.*, 2019), and even fewer studies take into account the match between daily schedule in pollen presentation and stigma receptivity as well as pollinator activity (Albrecht *et al.*, 2012). Through a quantitative estimation of pollinator performance, the present study showed that native bumblebees were much more efficient than exotic honeybees and native leafcutter bees, as they contributed the most of the seeds set per unit time. The better performance of bumblebees resulted from its greater overlap between the pattern of visitation frequency and the daily variation of stigma receptivity in the population of *L. floribunda*. Despite the higher number of visits accomplished by honeybees, the greater part occurred when most stigmas were still closed.

Consequently, bees mostly removed pollen and were less effective in pollen deposition. On the other hand, leafcutter bees were less predictable among years with a very low visitation frequency and effectiveness. Hence, we predict that bumblebees are likely responsible for the majority of seed production in the population. Analyses revealed that neither visitation frequency nor pollen removal or deposition alone were enough to provide a reliable estimate of pollinator performance. Differences in performance between pollinators were larger when the majority of stigmas were receptive (between 11:00 h and 13:00 h) than when it was estimated through the entire period of pollinators activity. These results highlight the importance of a more accurate distinction among the different events during the pollination process and how each pollinator contributes to final plant fitness (Valverde *et al.*, 2019). Thus, combining floral phenology with pollinator activity provided a more realistic estimation of pollinator performance.

Pollinator activity usually depends on climatic conditions throughout the day and availability of resources (Stone *et al.*, 1999). Difference in the temporal pattern of visitation to flowers among flower visitors has been attributed to a distinct temperature–foraging activity ratio (Free, 1968). However, pollinators that share the same floral resource can compete promoting displacement of foraging patterns (Valido *et al.*, 2019). Previous studies indicate that wild bees show lower visitation rates and lower fidelity to individual plant species over time when competing with honeybees (Isaacs and Kirk, 2010). In addition, honeybees overabundance leads to a re-assembly of plant-pollinator interactions through increased competition with other pollinator species (Magrath *et al.*, 2017). Although we cannot rule out the possibility that bumblebees and honeybees competed in our studied population, the stronger match between bumblebees activity and the pattern of nectar production suggest that a negative effect of honeybees on bumblebees through competitive interactions may not explain the observed

pattern. Nevertheless, we can not rule out the possibility that the lower effectiveness of leafcutter bees resulted from competitive interactions with honeybees and/or bumblebees.

Parallel to the generalized decline in pollinator availability due to anthropogenic alteration of natural environments (Winfrey *et al.*, 2009), honeybees have distributed almost all over the globe affecting the structure and functioning of natural pollination system (Ollerton *et al.*, 2012; Aizen and Harder, 2009). This change in the pollination ecology is expected to alter fitness benefits of plants, population genetic structure and future evolution of plant reproductive traits (Magrach *et al.*, 2017). The majority of progeny (approx. 75%) of *L. floribunda* resulted from outcrossing suggesting that pollinators play a leading role in the species reproductive success. A previous study in the same population of *L. floribunda* showed that only one visit during the male phase of flowers strongly decreases autonomous selfing by pollen removal (Roldan and Ashworth, 2018). This finding strengthens the importance of pollinators and protandry in promoting outcrossing. Our results show that honeybees may impose a higher cost to plant reproduction than bumblebees, because they produced fewer seeds per visit and consume both nectar and pollen. In addition, it is likely that the longer and more frequent intra-plant visits accomplished by honeybees can promote selfing through geitonogamy over outcrossing (Ma *et al.*, 2018), thus, reducing offspring quality through limited pollen competition (Magrach *et al.*, 2017). However, the accumulation of pollen by honeybees before stigmas are receptive may promote a more diverse pollen load reducing the likelihood of geitonogamy at anthesis (Roldan and Ashworth, 2018). Thus future studies should examine the relative contribution of each pollinator to the population outcrossing rate (Valverde *et al.*, 2019) and the costs of reproduction.

A recent study demonstrated that the more effective recently arrived invasive *Bombus terrestris* to a Chilean population of *Erythranthe lutea* (Phrymaceae) modified selection patterns acting on floral traits (Medel *et al.*, 2018). Our results indicate that the production of seeds by

bumblebees not only costs fewer pollen grains for plants but also less investment in the floral display than honeybees. Future changes in pollinators abundance may affect *L. floribunda* floral evolution (Ashworth *et al.*, 2015). Whereas bumblebees likely select for the maintenance of protandry, honeybees will probably select for its reduction. If honeybees were to increase even more their current abundance, new selective pressures are expected to act on floral display, nectar production and dichogamy. Thus monitoring selection patterns on floral traits and pollinators abundance will help to predict floral evolution in a changing world.

This comparative study provides new evidence of the factors that play a critical role when estimating pollinators performance. Our results highlight the importance of a more precise distinction among the events during the pollination process and their contribution to final plant fitness. We suggest that combining floral phenology with pollinator activity provide a more realistic model of pollinator performance. Once the best pollinator is identified, the population genetic consequences and the costs of reproduction under different pollination environments can be estimated.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Information S1: Molecular techniques details and microsat features for the multilocus population outcrossing rate (t) estimation in *Lepechinia floribunda*.

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Table 1. Summary of metrics used to study the pollination performance in *Lepechinia floribunda* population.

Measured	Definition	Abbreviation	References
Visitation Rate	Total visits per species per minute of observation	Vr	Herrera, 1989
Relative Visits	Total visits per species over the total observed flowers	Rv	Herrera, 1989
Visitor relative abundance	Percentage of individuals of a given species in the relation of the total number of individuals observed	Ra	This study
Visitation Frequency	The proportion of visited flowers per species over time per hour	Vf	Dafni et al., 2005
Handling time	The time between arrival and departure from a flower by an insect	Vd	Zych et al., 2013
Pollen Removal	The difference in the number of pollen grains recorded between non-visited and visited flowers	Pr	This study
Pollen Deposition	The difference in the number of pollen grains on the receptive inner blades of the stigmatic lobules between non-visited and visited flowers	Pd	This study
Fruit/flower	The proportion of flower setting fruits per plant after one visit	Fs	Ma et al., 2018
Seed/fruit	Number of mature seeds per fruit after one visit	Ss	Ma et al., 2018
Pollen exportation effectiveness	Number of pollen grains removed from anthers multiplied by the visitation frequency: remotion per visit per hour	D _{te}	This study following Ne'eman et al., 2010
Pollen deposition effectiveness	Number of pollen grains deposited in the stigma multiplied by the visitation frequency: deposition per visit per hour	D _{td}	Ne'eman et al., 2010
Pollination efficiency	Number of seeds produced after one visit divided the maximum seed set potential of the flower (s), multiplied by the visitation frequency: seeds set per visit per hour	P _E	Ne'eman et al., 2010

Table 2. Foraging behaviour throughout the day by flower visitors of *Lepechinia floribunda* during two consecutive seasons. Ra: Relative abundance, Vr: visitation rate (visits/minute), Rv: Relative visits (Visits/flower). In bold indicated the most abundant flower visitor

Flower visitor		2013-2014			2014-2015			Average		
		Ra	Vr	Rv	Ra	Vr	Rv	Ra	Vr	Rv
Morning (9:00-12:00)	<i>Apis mellifera</i>	70.00	0.39	0.22	49.33	1.42	0.63	59.67	0.90	0.43
	<i>Chlorostilbon lucidus</i>	10.00	0.17	0.10	-	-	-	-	-	-
	<i>Centris tricolor</i>	6.67	0.03	0.02	1.72	0.33	0.00	4.20	0.18	0.01
	<i>Bombus pauloensis</i>	3.33	0.02	0.01	20.74	0.69	0.28	12.04	0.35	0.14
	<i>Bombus opifex</i>	-	-	-	7.24	0.37	0.13	-	-	-
	<i>Megachile sp.</i>	6.67	0.02	0.01	16.2	0.49	0.27	11.44	0.25	0.14
	<i>Augochlora sp.</i>	3.33	0.01	0.01	1.35	0.26	0.10	2.34	0.14	0.05
	Average	-	0.11	0.06	-	0.53	0.19	-	0.32	0.13
Noon (12:00-14:30)	<i>Apis mellifera</i>	68.42	0.36	0.25	33.71	0.31	0.13	51.07	0.34	0.19
	<i>Chlorostilbon lucidus</i>	2.63	0.01	0.01	-	-	-	-	-	-
	<i>Bombus pauloensis</i>	2.63	0.01	0.01	14.29	0.41	0.19	8.46	0.21	0.10
	<i>Bombus opifex</i>	-	-	-	9.14	0.35	0.17	-	-	-
	<i>Megachile sp.</i>	2.63	0.01	0.00	17.71	0.24	0.19	10.17	0.12	0.10
	<i>Augochlora sp.</i>	18.42	0.07	0.05	16.57	0.22	0.10	17.50	0.15	0.07
	<i>Dialictus sp.</i>	5.26	0.01	0.01	1.14	0.07	0.04	3.20	0.04	0.02
	<i>Centris tricolor</i>	-	-	-	6.29	0.22	0.00	-	-	-
Average	-	0.08	0.05	-	0.29	0.13	-	0.18	0.09	
Afternoon (14:30-17:30)	<i>Apis mellifera</i>	52.63	0.17	0.10	68.12	0.27	0.17	60.38	0.22	0.14
	<i>Chlorostilbon lucidus</i>	10.53	0.04	0.02	-	-	-	-	-	-
	<i>Megachile sp.</i>	-	-	-	10.03	0.27	0.18	10.03	0.27	0.18
	<i>Bombus pauloensis</i>	26.32	0.09	0.05	11.31	0.48	0.15	18.82	0.29	0.10
	<i>Bombus opifex</i>	-	-	-	1.29	0.25	0.15	-	-	-
	<i>Dialictus sp.</i>	10.53	0.02	0.01	5.14	0.12	0.13	7.84	0.07	0.07
	<i>Centris tricolor</i>	-	-	-	4.11	0.2	0.00	4.11	0.20	0.00
	Average	-	0.08	0.05	-	0.26	0.11	-	0.17	0.08

Legends

Figure 1. Schematic representation of the relationships among floral traits and interaction traits determining pollination effectiveness, efficiency and performance.

Figure 2. (A) Nectar volume (white dots) and concentration (gray dots) over time in a random sample of flowers in a natural population of *Lepechinia floribunda*. **(B)** Percent of receptive and non-receptive stigmas (bars) and sugar production (black dots; nectar volume x nectar concentration). Sample size per harvest is indicated above each hour. Non-parametric regression analysis and a cubic spline adjustment were performed to show the sugar production pattern through time. Dotted lines show ± 1 Bayesian standard error interval.

Figure 3. Components of pollination performance for *Apis mellifera* (white), *Bombus spp.* (gray) and *Megachile sp.* (Black) in *Lepechinia floribunda*. Left panels represent average values and right panels correspond to the per hour estimates. **(A-B)** Visitation frequency, **(C-D)** Handling time, **(E-F)** Pollen removal (difference in the number of pollen grains recorded between non-visited and visited flowers expressed as percentage), **(G-H)** Pollen deposition (logarithm of the difference in the number of pollen grains on the receptive inner blades of the stigmatic lobules among visited flowers). Vertical lines within the left panels represent standard errors. Sample size per hour of data recording is indicated above each hour. NS, non-significant difference; * $p < 0.05$; ** $p < 0.01$.

Figure 4. (A) Percent of mean number of fruits/flowers, and **(B)** seeds/flower formed after a visit of *Apis mellifera* or *Bombus spp.* in a natural population of *Lepechinia floribunda*. Vertical lines in each bar represent standard errors. ** $p < 0.001$.

Figure 5. Pollination effectiveness of *Apis mellifera* (light grey), *Bombus spp.* (dark grey) and *Megachile sp.* (Black). in *Lepechinia floribunda*. **(A)** Pollen exportation effectiveness (D_{te}). **(B)** Pollen deposition effectiveness (D_{td}). Left panels show the daily variation in pollination effectiveness for each floral visitor. Standard error was obtained after 1000 bootstrap samples for each hour. Right bar plots represent the weighted average of D_t throughout the complete observation period (8:00 - 13:00 pm), and after more than 70% stigmas were receptive in the population (11:00 - 13:00 pm). Vertical lines represent standard error after 1000 bootstrap samples. NS, non-significant difference; * $p < 0.05$; *** $p < 0.001$.

Figure 6. (A) Mean pollination efficiency of *Apis mellifera* (white bar) and *Bombus spp.* (grey bar). Vertical lines represent standard errors obtained after 1000 bootstrap samples. **(B)** Frequency distribution of pollination efficiency for each pollinator after the bootstrapping process. * $p < 0.05$.

Figure 1.

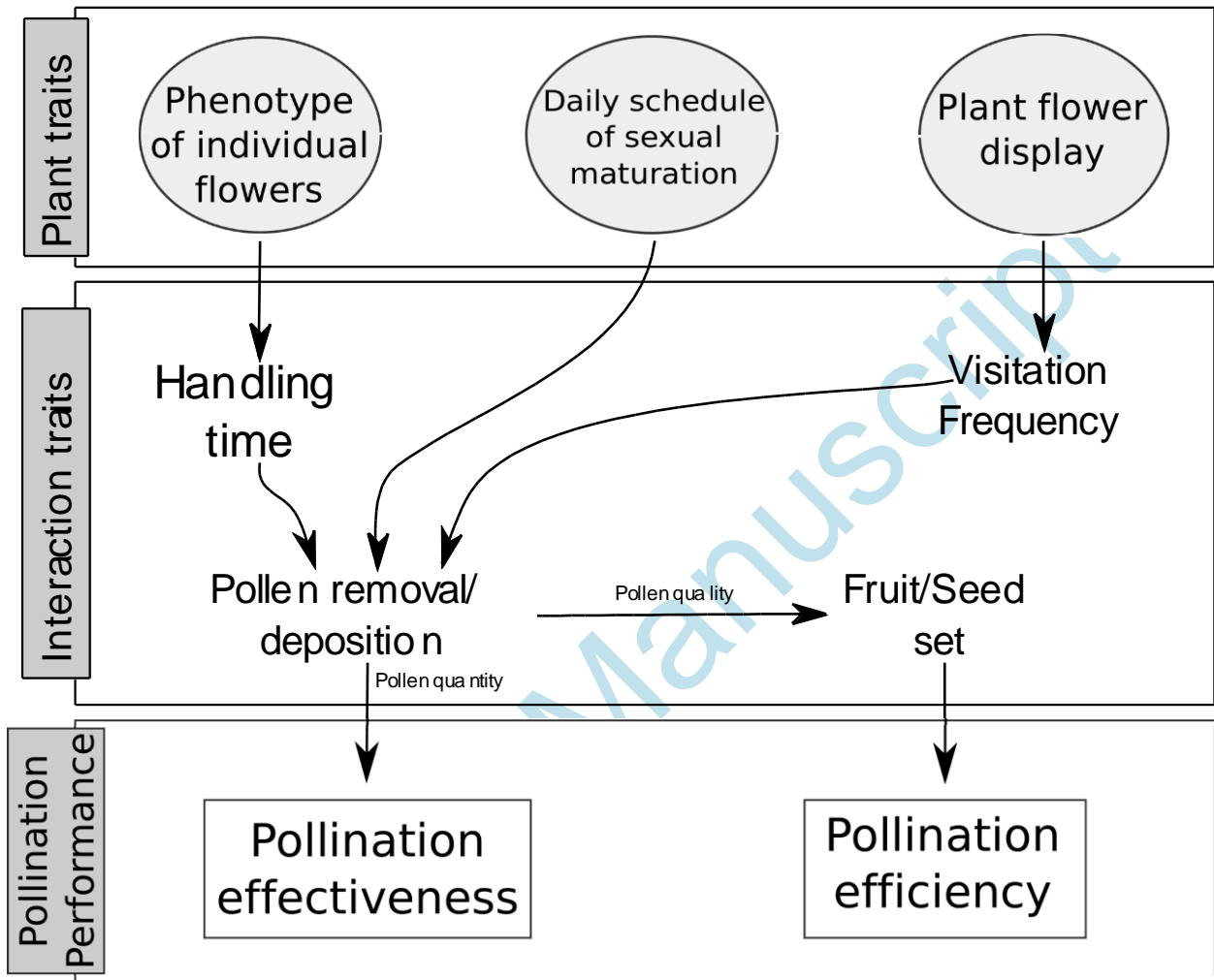


Figure 2.

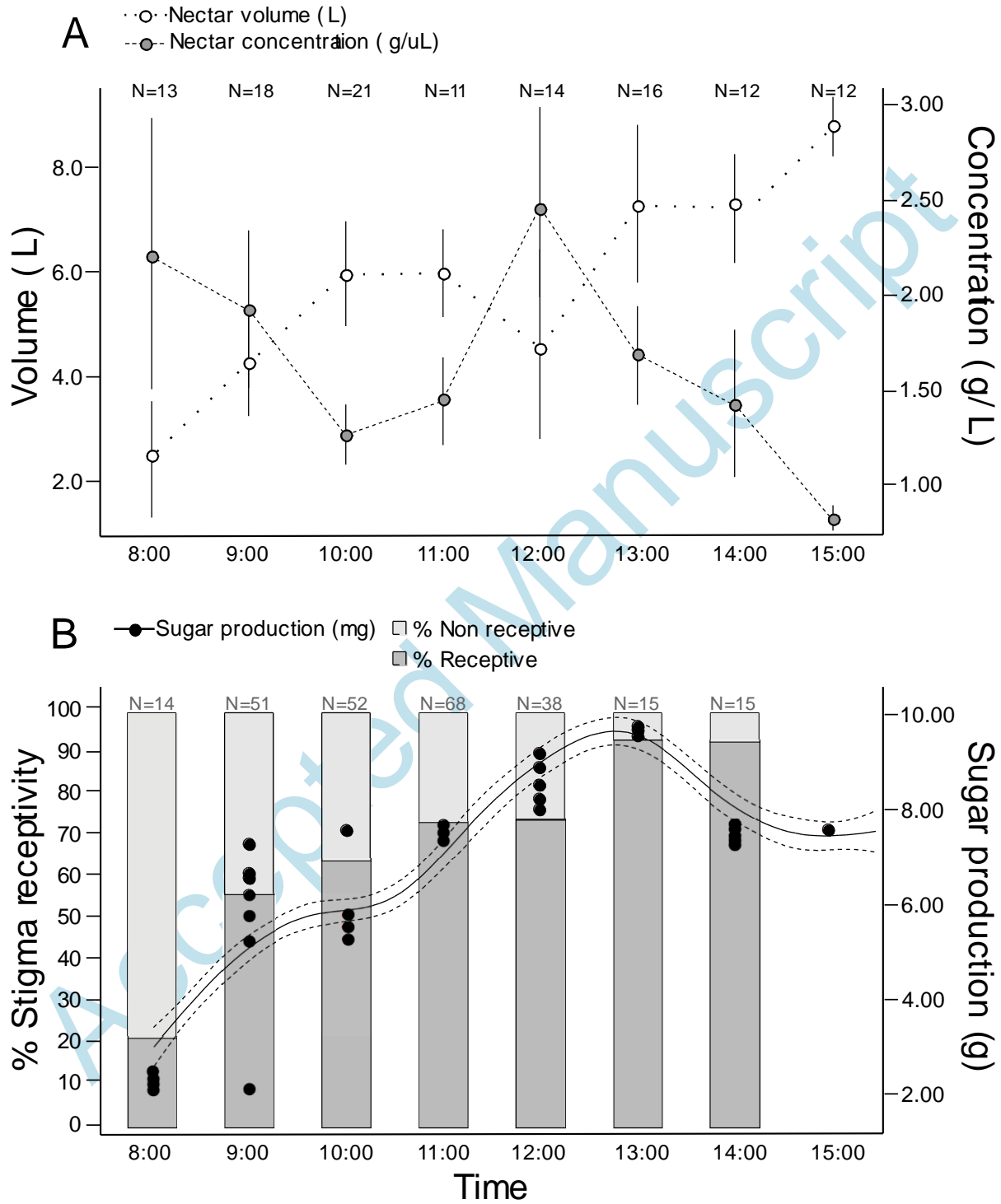


Figure 3.

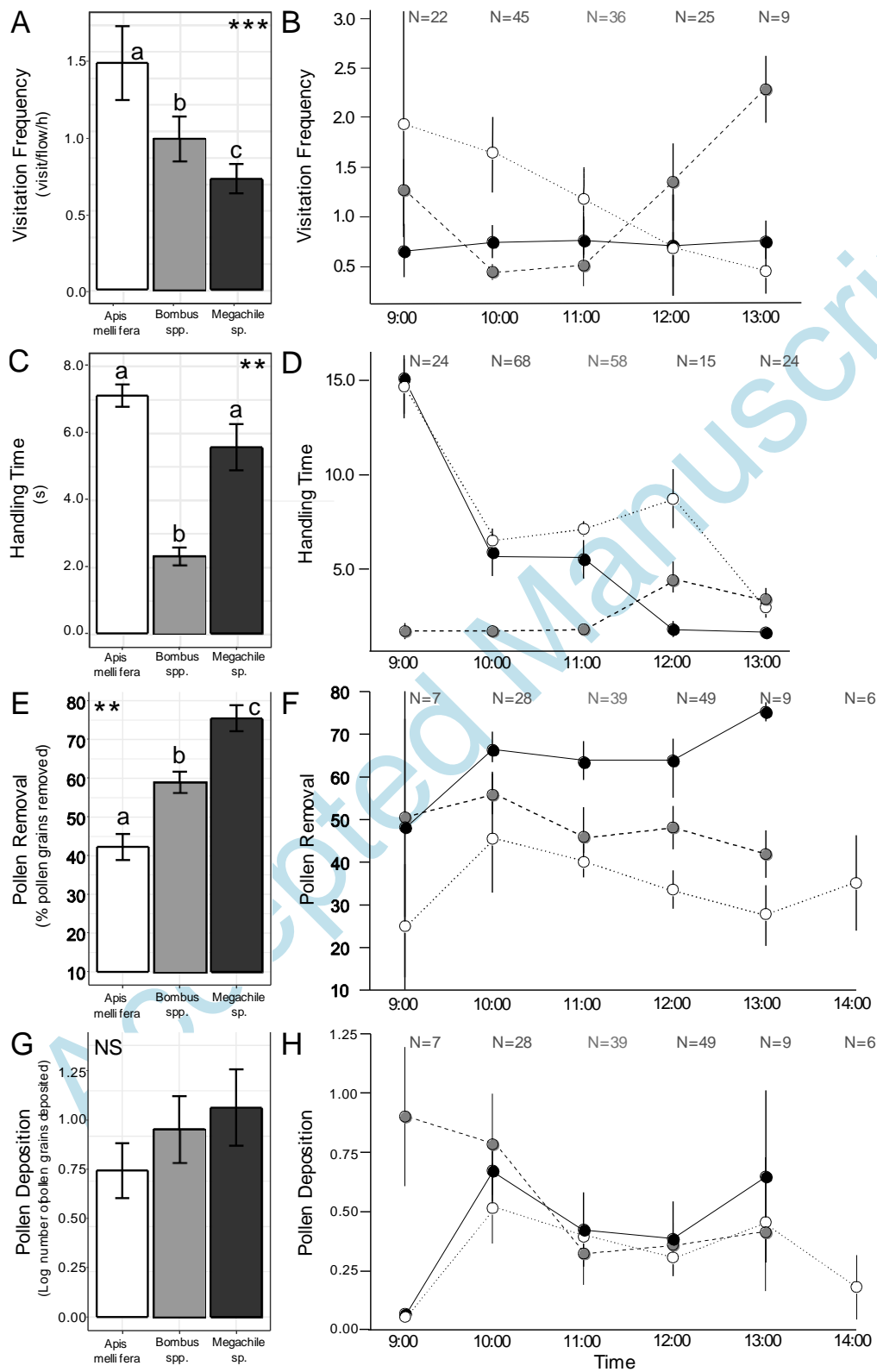


Figure 4.

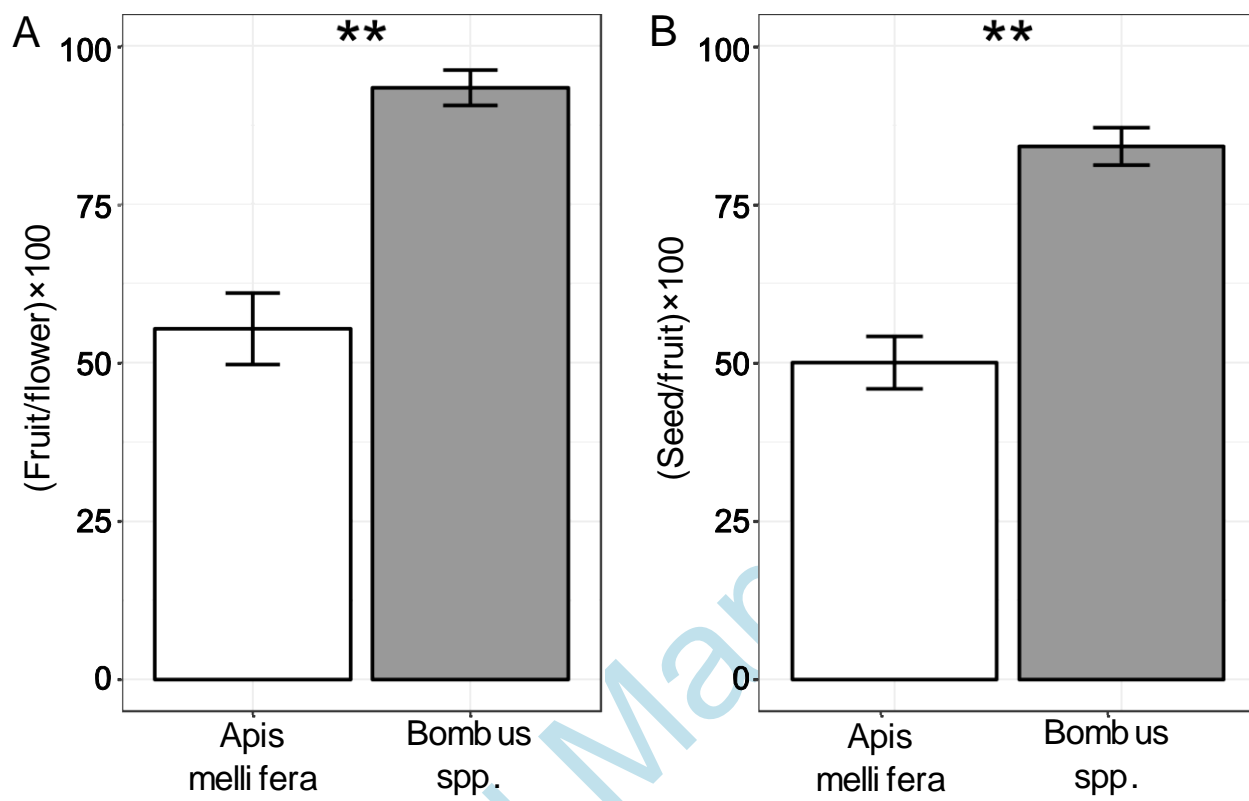


Figure 5.

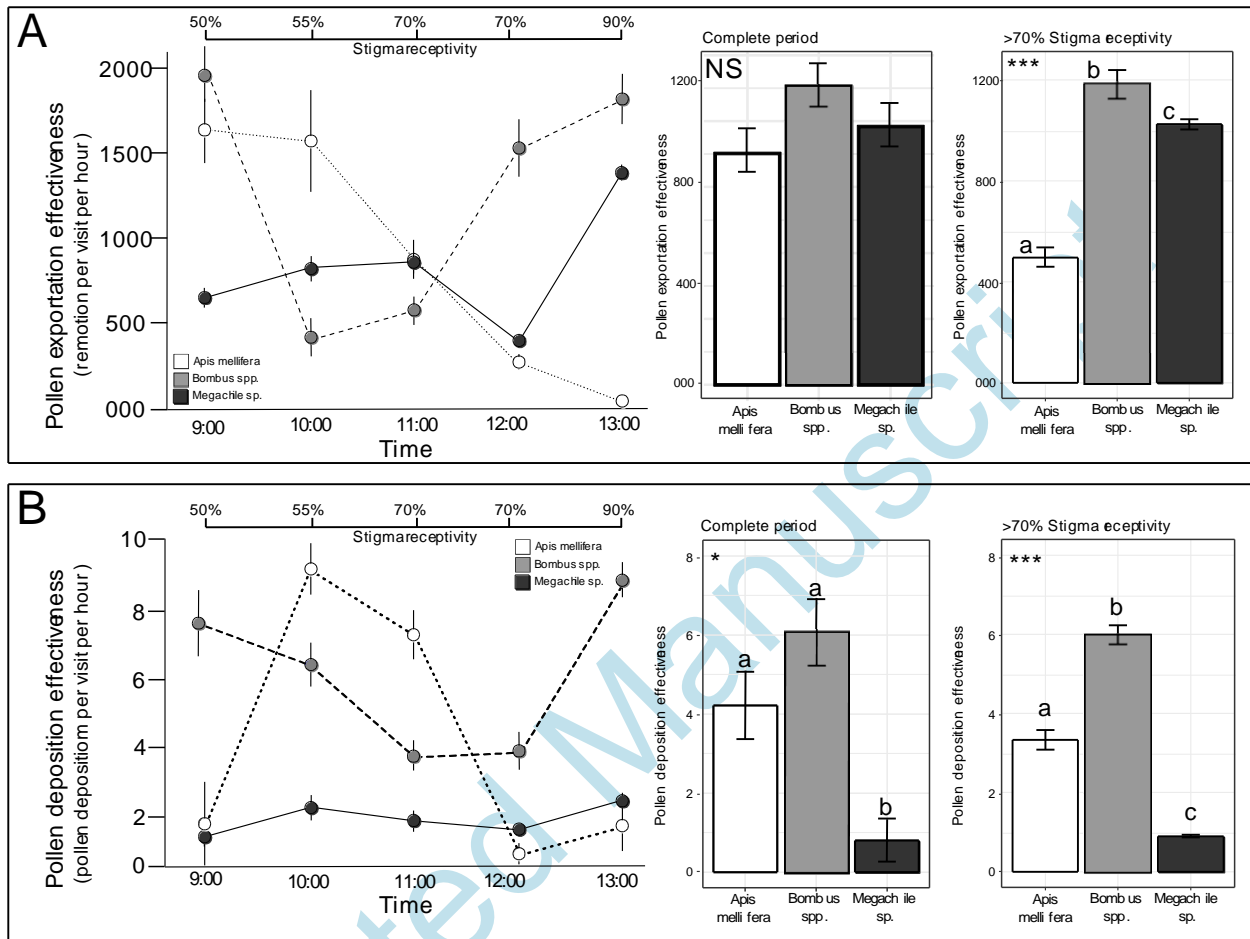


Figure 6.

