

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 being proposed decades ago, the most important pollinators are still mainly identified using the frequency of visits to flowers. This shortcoming results in a gap between the characterization of the flower visitors of a plant species and a reliable estimation of the plant fitness consequences of the mutualistic interaction. The performance of a mutualistic visitor depends on its abundance, behaviour, effectiveness (pollen removal and deposition per unit time) and efficiency (seed set per unit time) conditioned by the temporal matching between pollinator activity and temporal patterns of maturation of the sexual functions of flowers. Although there have been 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 the flower biology and mating system of the sub-shrub *Lepechinia floribunda* (Lamiaceae) to provide a daily quantitative estimation of performance (effectiveness and efficiency) of the more abundant pollinators, i.e. native bumble-bees (*Bombus* spp.) and leafcutter bees (*Megachile* sp.), and the exotic honey-bee (*Apis mellifera*).
- **Key Results** Unlike honey-bees or leafcutter bees, native bumble-bees matched the daily pattern of nectar production and stigma receptivity, and showed higher effectiveness and efficiency. Despite the overabundance of honey-bees, visits occurred mainly when stigmas were not receptive, thus reducing the honey-bees' overall performance.
- **Conclusions** Bumble-bees appear to be the most important pollinators and potential historical mediators of reproductive trait evolution in *L. floribunda*. Because the production of seeds by bumble-bees involved fewer pollen grains for plants and less investment in floral display than honey-bees, contemporary and expected changes in pollinator abundance may affect future *L. floribunda* floral evolution. If bumble-bees were to be further displaced by anthropogenic disturbance or by competition with honey-bees, their lower efficiency will select for a larger floral display increasing reproductive costs. This scenario may also impose selection to reduce dichogamy to match honey-bee foraging activity.

Key Words: Foraging behaviour, *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 the fact that recent theoretical and methodological proposals argue for a more precise estimation of pollinator 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 pollinator activity is concentrated during only one phase of sexual maturation, either when anthers open or when 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 Fig. 1. The 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 at a flower, morphological matching between a pollinator and floral architecture, together with pollinator behaviour, 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 into the final performance of each pollinator species. In addition, within a population, pollen movement among plants and the resulting outcrossing rate depend on the co-ordination between the period of pollinator activity and the timing of maturation of sexual phases among flowers (Herrera, 2000). Hence, the variation in the number of receptive flowers during 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 pollinator activity affect pollination effectiveness and efficiency (Fig. 1; Herrera, 1987, 2000), 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, bumble-bees and the cosmopolitan honey-bee *Apis mellifera* (Roldan and Ashworth, 2018). As native bees have a longer history of interaction with the selected plant species than honey-bees, it is expected that native bees will show greater effectiveness and/or efficiency than honey-bees. 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 by a higher visitation frequency. In the present study, we (1) describe the floral biology of *L. floribunda* (daily patterns of both nectar secretion and stigma receptivity); (2) examine the relevance of pollinators for seed production through outcrossing rate (t) estimation; (3) assess relative abundance, visitation rate and relative visits of the whole community of floral visitors; and (4) compare pollination effectiveness (i.e. the 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 sub-shrub native of the montane forests in Argentina, Bolivia and Peru commonly found in dry open habitats from 500 to 3500 m 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 1 day and present bifid stigmas and four ovules. They are incomplete protandrous (anthers mature before stigmas), and can self-fertilize autonomously (Roldan and Ashworth, 2018).

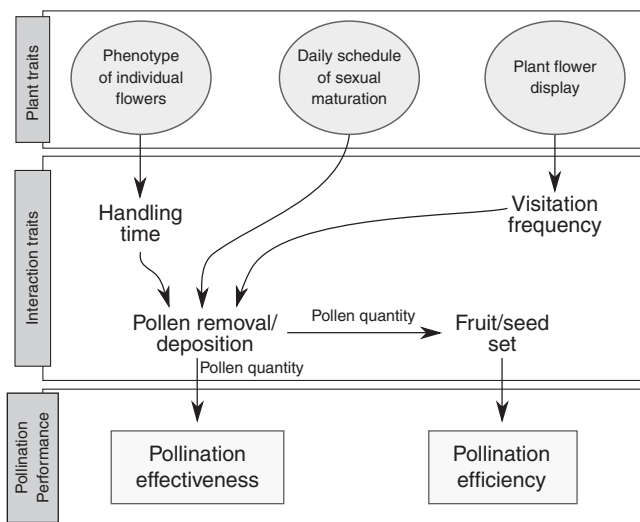


FIG. 1. Schematic representation of the relationships among floral traits and interaction traits determining pollination effectiveness, efficiency and performance.

Fruits mature approx. 3 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 07.00 h. Opened flowers were harvested every hour (12–21 bagged flowers per hour) from 08.00 h to 15.00 h when nectar production dropped. Nectar volume (μL) and concentration ($\mu\text{g } \mu\text{L}^{-1}$) were recorded using 1 or 5 μL micro-caps and a temperature-compensated hand refractometer (concentration range 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 1 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 microsatellite 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 and its period of greatest activity, and determined the abundance of its members by recording mean visitation rate (visits per minute; Vr), relative visits (visits per observed flowers; Rv) and relative abundance (Ra; Table 1) of each floral visitor species during two consecutive reproductive seasons. Direct observations of visitors were made between 08.00 h and 17.00 h, in periods of 15 min per plant, during 10 d, 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 the Results and Table 2).

Following our previous characterization, subsequent estimations of pollinator performance were obtained for the honey-bee (*Apis mellifera*), leafcutter bees (*Megachile* sp.) and a set of two functionally equivalent native bumble-bee

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 relation to the total number of individuals observed	Ra	This study
Visitation frequency	The proportion of visited flowers per species per hour	Vf	Dafni <i>et al.</i> (2005)
Handling time	The time between arrival and departure from a flower by an insect	Ht	Zych <i>et al.</i> (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 <i>et al.</i> (2018)
Seed/fruit	Number of mature seeds per fruit after one visit	Ss	Ma <i>et al.</i> (2018)
Pollen exportation effectiveness	Number of pollen grains removed from anthers multiplied by the visitation frequency: removal per visit per hour	D _{ie}	This study following Ne'eman <i>et al.</i> (2010)
Pollen deposition effectiveness	Number of pollen grains deposited in the stigma multiplied by the visitation frequency: deposition per visit per hour	D _{id}	Ne'eman <i>et al.</i> (2010)
Pollination efficiency	Number of seeds produced after one visit divided by the maximum seed set potential of the flower (s), multiplied by the visitation frequency: seeds set per visit per hour	P _E	Ne'eman <i>et al.</i> (2010)

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* and *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 09.00 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 this was mounted in a drop of stained glycerine jelly previously held on a slide (Baranzelli et al., 2014). The 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 numbers of pollen grains in the anthers and on the stigmas were counted using digital images from slides taken at $\times 10$ 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 ($\text{Pr}_{\text{unvisited flowers}} - \text{Pr}_{\text{visited flowers}}$). 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

TABLE 2. Foraging behaviour throughout the day by flower visitors of *Lepechinia floribunda* during two consecutive seasons

	Flower visitor	2013–2014			2014–2015			Average		
		Ra	Vr	Rv	Ra	Vr	Rv	Ra	Vr	Rv
Morning (09.00–12.00 h)	<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</i> sp.	6.67	0.02	0.01	16.2	0.49	0.27	11.44	0.25	0.14
	<i>Augochlora</i> sp.	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 h)	<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</i> sp.	2.63	0.01	0.00	17.71	0.24	0.19	10.17	0.12	0.10
	<i>Augochlora</i> sp.	18.42	0.07	0.05	16.57	0.22	0.10	17.50	0.15	0.07
	<i>Dialictus</i> sp.	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 h)	<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</i> sp.	–	–	–	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</i> sp.	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

Ra, relative abundance; Vr, visitation rate (visits min^{-1}); Rv, relative visits (visits per flower).

The most abundant flower visitor is indicated in bold.

were performed for every 1 h interval between 09.00 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 per flower (Fs) and seed per 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 the Results), it presents very low effectiveness and 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 mid-day (see the Results), pollination effectiveness was calculated for every 1 h interval during pollinator activity to obtain the daily variation pattern and more accurate estimation of the response variable. Following the framework of Ne'eman et al. (2010), pollination effectiveness per time unit (D_t) for pollinators was estimated as the product of its visitation frequency (Vf) and the number of pollen grains removed from anthers (Pr) or deposited on the stigmas (Pd), as indicated below:

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

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

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:

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

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 the `gam` function (package: `mgcv`; Wood, 2006) in R. Smoothing parameters were obtained by minimizing the generalized cross-validation 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 (GLMMs; 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 and 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 Akaike information criterion (AIC) comparisons of models. For Vf, Ht, Pd and 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 pollinator activity, weighted mean effectiveness was obtained for each pollinator as $\sum_{t=0}^n D_t$ where n is the period of observation following Eqns (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 pollinator activity (09.00–13.00 h) and for the period when >70 % of the opened flowers had their stigma receptive in the population (11.00–13.00 h; see Fig. 2). Comparisons were performed using linear models with the `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 Eqn (3), was performed using zero-inflated negative binomial (ZINB) models to consider overdispersion and excess of zeros in the data. The ZINB model was tested as suggested by Zuur et al. (2009; see the Results), using the `zeroinfl` function (package: `pscl`; Zeileis et al., 2008).

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

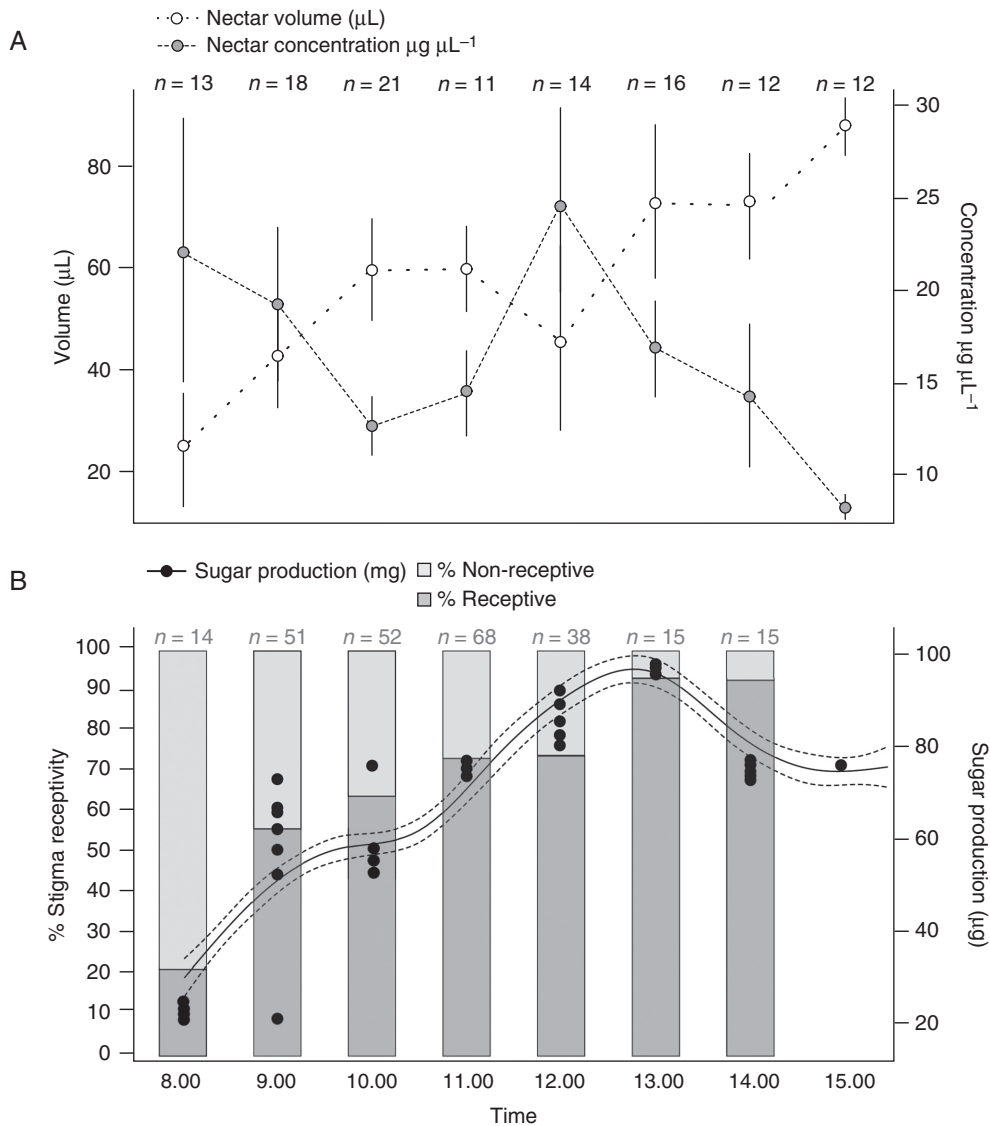


FIG. 2. (A) Nectar volume and concentration over time in a random sample of flowers in a natural population of *Lepechinia floribunda*. (B) Percentage of receptive and non-receptive stigmas and sugar production (nectar volume \times nectar concentration). Sample size per harvest is indicated above each 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.

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 ($\mu\text{g } \mu\text{L}^{-1}$). Nectar volume continuously increased throughout the day, reaching 9.4 ± 1.6 μL before 15.00 h. There were significant differences in nectar volume across time ($F = 3.794$; $P = 0.001$), in particular among the first hours of the morning (08.00 h and 9.00 h), mid-day (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 h (2.5 ± 1.4 $\mu\text{g } \mu\text{L}^{-1}$), and a minimum concentration at 15.00 h (0.8 ± 0.1 $\mu\text{g } \mu\text{L}^{-1}$; 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 of nectar with 1.6 ± 0.1 $\mu\text{g } \mu\text{L}^{-1}$ sugar per flower. Sugar production increased throughout the day (explained deviance = 79.7%; $P < 0.0001$), with a peak at around 12.00–13.00 h (9.4 ± 0.5 μg ; Fig. 2B).

The proportion of receptive stigmas followed a similar trend to volume and sugar production. The first receptive stigmas were observed early in the morning at around 08.00 h but at low frequency (20%). It was not until 11.00 h that >70% of the observed flowers had their stigmas open with an angle of >90° that indicated receptiveness. By 13.00 h, >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 2–6 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 and Megachilidae), and one hummingbird, *Chlorostilbon lucidus* (Trochilidae; [Table 2](#)). Hummingbirds were observed only in one season (in 2013–2014), while other visitors appeared in both seasons ([Table 2](#)). The sweat bee *Dialictus* sp. appeared at noon and in the afternoon. Taking all floral visitors into account, the highest Vr and Rv were recorded in the morning (0.32 visits min^{-1} ; 0.13 visits per flower) and early afternoon (0.18 visits min^{-1} ; 0.09 visits per flower). All floral visitors were observed collecting either pollen, nectar or both. Together, bumble-bees, leafcutter bees and honey-bees represented >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 per flower h^{-1}) than for *Bombus* spp. (0.83 ± 0.13 visits per flower h^{-1}) or *Megachile* sp. (0.62 ± 0.08 visits per flower h^{-1} ; $F = 4.67$; $P = 0.0013$; [Fig. 3A](#)). *Apis mellifera* was the main visitor until 11.00 h, 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 per flower) than *Megachile* sp. (5.56 ± 3.53 s per flower) and *A. mellifera* (7.09 ± 3.70 s per flower); [Fig. 3C](#)). While *A. mellifera* and *Megachile* sp. showed a consistent reduction throughout the day in the handling time per flower, *Bombus* spp. were 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 ($\Delta\text{AIC} = 3130$; $P = 0.007$), 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 ($\Delta\text{AIC} = 9.478$; $P = 0.002$), explaining 29 % of the variation.

Fruit set and seed set

Among tagged plants, fruit per flower (Fs) varied from 33 to 100 %, and seed per 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 (09.00–13.00 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. with respect to *Megachile* sp. were detected ($F = 3.398$; $P = 0.043$; [Fig. 5A](#)). Nevertheless, at the time of the day when >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 mid-day, *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 (P_E) for this floral visitor. The P_E of *Bombus* spp. per hour was significantly higher than that of *A. mellifera* (0.38 ± 0.01 vs. 0.14 ± 0.01) ($\beta = -2.17$, s.e. = 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 fewer 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 honey-bee than by a bumble-bee. ([Fig. 6B](#)).

DISCUSSION

We were able to disentangle pollinator performance of honey-bees (*A. mellifera*), bumble-bees (*Bombus* spp.) and leafcutter bees (*Megachile* sp.) through quantitative estimation of effectiveness and efficiency (*sensu* Ne'eman *et al.*, 2010), despite

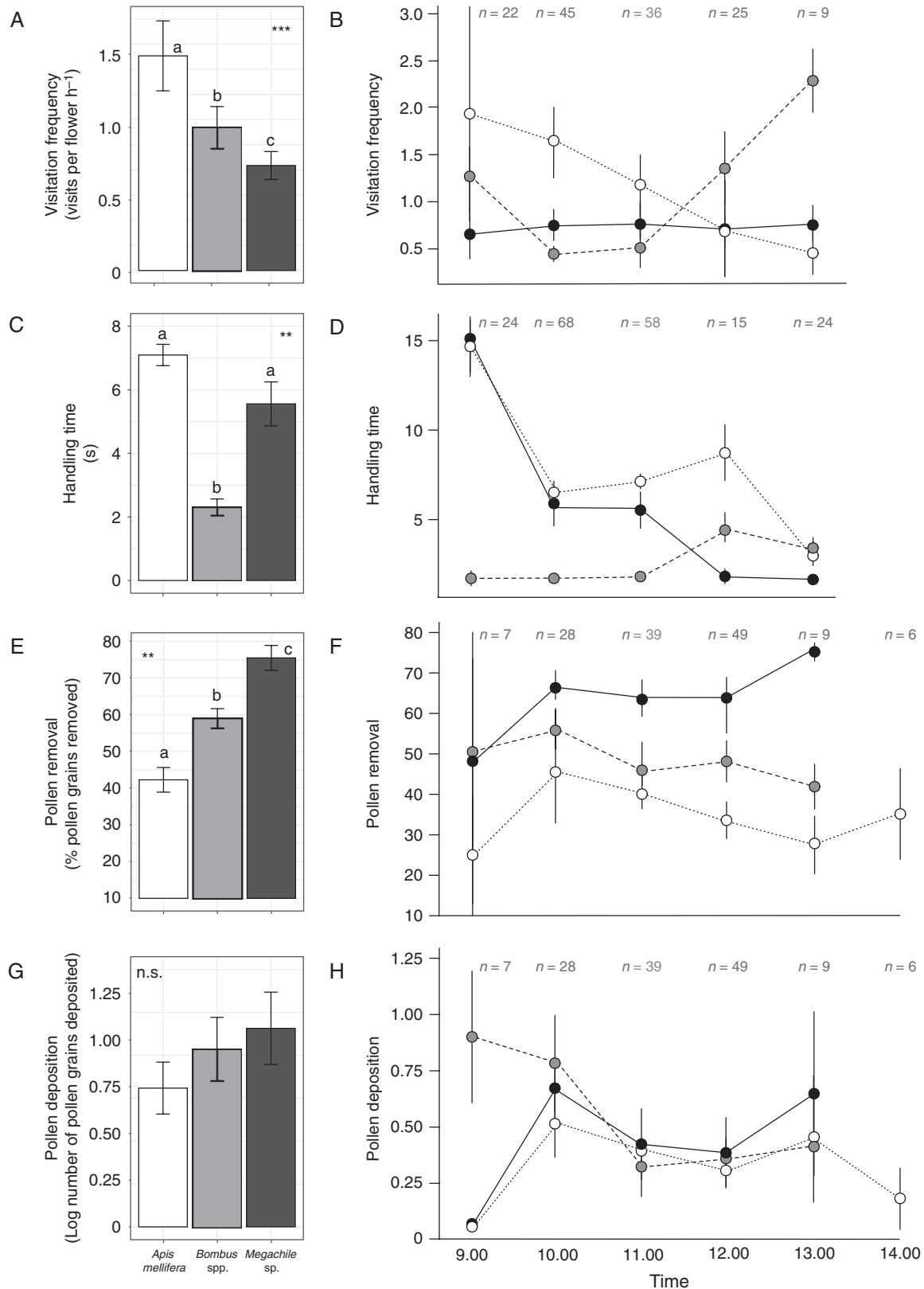


FIG. 3. Components of pollination performance for *Apis mellifera*, *Bombus* spp. and *Megachile* sp. in *Lepechinia floribunda*. Left panels represent average values and right panels correspond to the per hour estimates. (A and B) Visitation frequency, (C and D) handling time, (E–F) pollen removal (difference in the number of pollen grains recorded between non-visited and visited flowers expressed as a percentage) and (G and 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 the s.e. Sample size per hour of data recording is indicated above each hour. n.s., non-significant difference; * $P < 0.05$; ** $P < 0.01$.

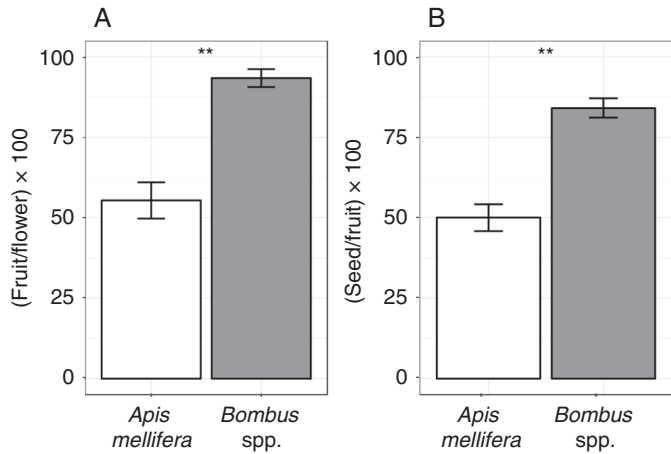


FIG. 4. (A) Percentage of mean number of fruits per flowers, and (B) seeds per flower formed after a visit of *Apis mellifera* or *Bombus* spp. in a natural population of *Lepechinia floribunda*. Vertical lines in each bar represent the s.e. ** $P < 0.001$.

these pollinator groups belonging to the same functional guild. As expected, honey-bees 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 bumble-bees over honey-bees and leafcutter bees. The results demonstrate that the higher visitation frequency of honey-bees was not enough to surpass the higher effectiveness and efficiency of the native bumble-bees. Thus, bumble-bees are probably the most important pollinators affecting the evolution of reproductive traits in *L. floribunda* through their positive effect on plant fitness. Under present levels of performance of honey-bees, plants would have to produce three times more flowers, or honey-bees would have to double their visitation frequency, to attain a seed production per hour as high as is attained by the native bumble-bees. This scenario may impose a strong selection favouring an increase in floral display

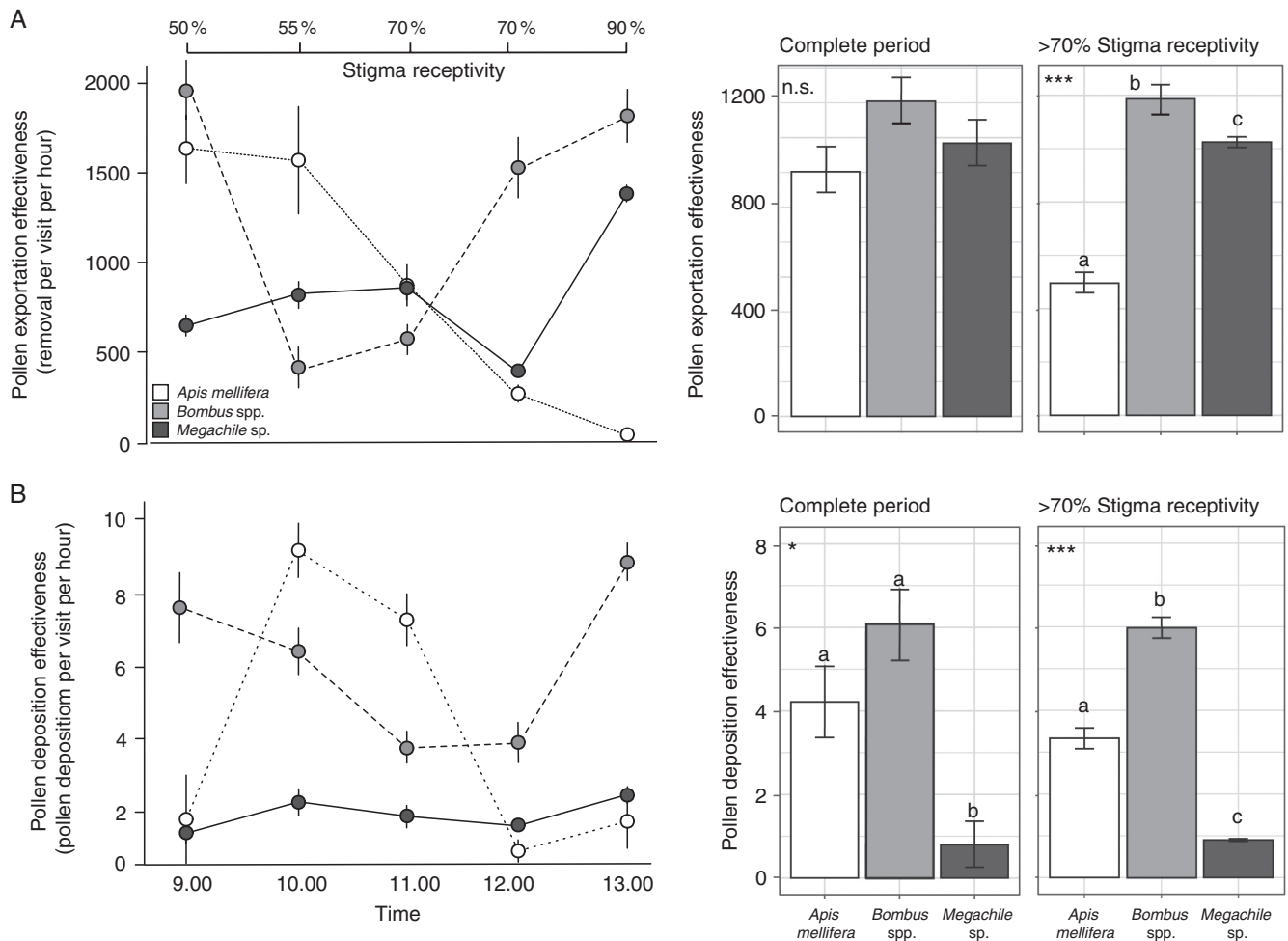


FIG. 5. Pollination effectiveness of *Apis mellifera*, *Bombus* spp. and *Megachile* sp. in *Lepechinia floribunda*. (A) Pollen exportation effectiveness (D_e). (B) Pollen deposition effectiveness (D_i). Left panels show the daily variation in pollination effectiveness for each floral visitor. The s.e. was obtained after 1000 bootstrap samples for each hour. Right bar plots represent the weighted average of D_i throughout the complete observation period (08.00–13.00 h), and after >70 % of stigmas were receptive in the population (11.00–13.00 h). Vertical lines represent the s.e. after 1000 bootstrap samples. n.s., non-significant difference; * $P < 0.05$; *** $P < 0.001$.

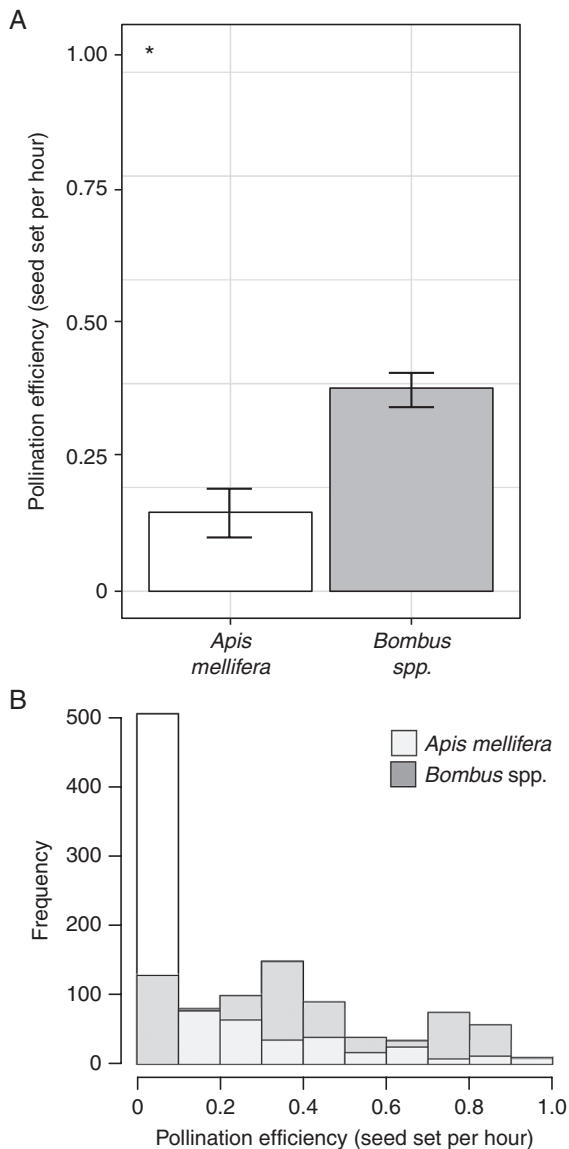


FIG. 6. (A) Mean pollination efficiency of *Apis mellifera* and *Bombus* spp. Vertical lines represent the s.e. obtained after 1000 bootstrap samples. (B) Frequency distribution of pollination efficiency for each pollinator after the bootstrapping process. * $P < 0.05$.

and a reduction in dichogamy to match the foraging of activity honey-bees during the day if bumble-bees were to be displaced by anthropogenic disturbance or by competition with honey-bees. Thus, replacement of functions is likely within the pollinator assemblage of *L. floribunda*, but this 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 percentage of open flowers with signals that pollinators had touched the stigma, the amount of pollen removal or deposition, or the handling time, among others (Ne'eman et al., 2010). However, few studies have combined any of these estimates with the 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 bumble-bees were much more efficient than exotic honey-bees and native leafcutter bees, as they contributed most of the seeds set per unit time. The better performance of bumble-bees resulted from the 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 honey-bees, 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 bumble-bees are likely to be 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 throughout the entire period of pollinator 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). A 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 in 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 honey-bees (Isaacs and Kirk, 2010). In addition, honey-bee 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 bumble-bees and honey-bees competed in our studied population, the stronger match between bumble-bee activity and the pattern of nectar production suggests that a negative effect of honey-bees on bumble-bees through competitive interactions may not explain the observed pattern. Nevertheless, we cannot rule out the possibility that the lower effectiveness of leafcutter bees resulted from competitive interactions with honey-bees and/or bumble-bees.

Parallel to the generalized decline in pollinator availability due to anthropogenic alteration of natural environments (Winfree et al., 2009), honey-bees have distributed almost all over the globe, affecting the structure and functioning of the natural pollination system (Aizen and Harder, 2009; Ollerton et al., 2012). This change in the pollination ecology is expected to alter fitness benefits of plants, population genetic structure and future evolution of plant reproductive traits (Magrath 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 honey-bees may impose a higher cost to plant reproduction than bumble-bees, 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 honey-bees can promote selfing through geitonogamy over outcrossing (Ma et al., 2018), thus reducing offspring quality through limited pollen competition (Magrath et al., 2017). However, the accumulation of pollen by honey-bees 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 recent arrival of 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 contribution of bumble-bees to seed production costs fewer pollen grains and less investment in the floral display for plants than honey-bee. Future changes in pollinator abundance may affect *L. floribunda* floral evolution (Ashworth et al., 2015). Whereas bumble-bees probably select for the maintenance of protandry, honey-bees will probably select for its reduction. If honey-bees were to increase their current abundance even more, new selective pressures are expected to act on floral display, nectar production and dichogamy. Thus monitoring selection patterns on floral traits and pollinator 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 pollinator 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 provides 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 Information S1: details of molecular techniques and microsatellite features for the multilocus population outcrossing rate (t) estimation in *Lepechinia floribunda*.

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