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Cuckoo male bumblebees perform slower and longer flower visits than free-living male and worker bumblebees

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Abstract. Cuckoo bumblebees are a monophyletic group within the genus *Bombus* and social parasites of free-living bumblebees, upon which they rely to rear their offspring. Cuckoo bumblebees lack the worker caste and visit flowers primarily for their own sustenance and do not collect pollen. Although different flower-visiting behaviours can be expected between cuckoo and free-living bumblebees due to different biological constraints, no study has yet quantified such differences. Here, we provide the first empirical evidence of different flower-visiting behaviours between cuckoo and free-living bumblebees. We recorded the flower-visiting behaviour of 350 individual bumblebees over two years in a wild population of the entomophilous plant *Gentiana lutea*, of which they are among the main pollinators. In cuckoo bumblebees (28.9% of the total), we only found males, while we found both workers and males in free-living bumblebees. Cuckoo bumblebees visited significantly more flowers for longer time periods than both free-living bumblebee workers and males within whorls, while differences at the whole-plant level were less marked. Free-living bumblebee males visited more flowers and performed slightly longer flower visits than workers. Behavioural differences between cuckoo male bumblebees and free-living bumblebee workers are likely related to different foraging needs, while differences between cuckoo and free-living bumblebee males may be caused by differences in colony development and a delayed mating period of free-living bumblebees. The longer visits made by cuckoo male bumblebees will likely negatively affect plant reproductive success through increased within-plant pollen flow.

Keywords. *Bombus*, flower-visiting behaviour, *Gentiana lutea*, plant-pollinator interactions, *Psithyrus*.

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Introduction

Bumblebees are primitively eusocial bees with an annual life cycle usually divided into three distinct phases. First, a solitary phase involving hibernation of mated gynes (i.e., reproductive females) followed by nest foundation (from late summer to early spring), then a multiplicative phase during which the founding queen produces workers (i.e., non-reproductive females, from mid-spring to mid-summer), and finally a reproductive phase during which males and gynes are produced (mid-late summer) (ALFORD 1975; LHOMME & HINES 2019).

As in other groups of social insects, social parasitism has evolved in several species of bumblebees, called cuckoo bumblebees (LHOMME & HINES 2019). There are currently 27 species of cuckoo bumblebees (subgenus *Psithyrus*) recognized worldwide (LHOMME *et al.* 2021). These species completely lack the worker caste (i.e., all the female individuals are fertile females) and do not have specialized structures on the hind legs (i.e., corbiculae) to collect pollen to feed their larvae. Consequently, females of cuckoo bumblebees usurp the nests of host social bumblebees (hereafter referred to as free-living bumblebees) almost always killing the host queen to force the host workers to rear their offspring (FISHER 1987).

Bumblebees are important pollinators of both wild flowering plants and crops (e.g., THOMSON & GOODELL 2001; PELLISSIER *et al.* 2012; FRÜND *et al.* 2013; ANDRIKOPOULOS & CANE 2018), and a few species are exploited commercially in agriculture (VELTHUIS & VAN DOORN 2006; GOULSON *et al.* 2008). As most pollinators, free-living bumblebees show intraspecific differences in foraging behaviour between sexes mainly in relation to offspring provisioning (SMITH *et al.* 2019). Workers visit flowers to collect pollen to feed the larvae and nectar for energy intake, while males visit flowers to seek nectar to fuel their flight and to look for mates (GOULSON 2010). Consequently, foraging females have generally high levels of flower constancy (RUSSELL *et al.* 2017), which increase pollination efficiency by transferring conspecific pollen between flowers of a same species (GOULSON 2010). However, male bumblebees can also inadvertently carry large amounts of pollen that can potentially contribute to pollination (JENNERSTEN *et al.* 1991; OSTEVIK *et al.* 2010; WOLF & MORITZ 2014). Although flower-visiting behaviour of free-living bumblebees has been widely studied, behaviour of cuckoo bumblebees remains almost unstudied (but see BATEMAN & RUDALL 2014), and to our knowledge there is still no assessment of similarities or differences between parasitic and non-parasitic bumblebees.

In this article, we investigate the flower-visiting behaviour of cuckoo and free-living bumblebees in a wild population of the entomophilous plant *Gentiana lutea* subsp. *symphyandra*, where both bumblebee types were abundant and among its most important pollinators. Specifically, we aim to answer two main questions: (1) do cuckoo and free-living bumblebees differ in the time spent visiting flowers and in the number of flowers visited, and (2) do cuckoo bumblebees display different foraging behaviours from those of males and workers of free-living bumblebees? An evaluation of the different flower-visiting behaviours between parasitic bumblebees and their hosts would increase our knowledge of their biology and allow us to estimate differences in pollination efficiency with implications for plant fitness.

Material and methods

Study site and species

This study was carried out in a natural population of *Gentiana lutea* L. subsp. *symphyandra* (Murb.) Hayek situated on the eastern face of Mount Grande (Northern Apennines; Bologna, Italy), within the Habitat Directive site “IT4050002 SIC-ZPS Corno alle Scale” (1380–1460 m a.s.l.; 44°8'57" N, 10°52'10" E). The study population is located in a steep clearing surrounded by a beech (*Fagus sylvatica* L.) forest.

Gentiana lutea subsp. *symphyandra* is a perennial herb growing on mountains of south-eastern Europe that flowers between June and July (ROSSI *et al.* 2015). The tall fertile stems bear several yellow flowers with wide-open corollas of about 4 cm in diameter (personal observation MG), grouped in pseudo-whorls (i.e., multiple flowers grouped tightly together around the stem, subtended by two opposite bracts; hereafter: whorls for simplicity) that flower sequentially from the bottom to the top. Flowers of *G. lutea* are functionally and ecologically generalized (*sensu* OLLERTON *et al.* 2007), being efficiently visited by several insect species belonging to four orders (Hymenoptera, Diptera, Coleoptera, Lepidoptera) with different physiological and energetic requirements (ROSSI *et al.* 2014). Among these, bumblebees have the highest pollinator performance, fidelity and visitation frequency in the studied population (ROSSI *et al.* 2014). Although *G. lutea* is a self-compatible species, the partial flower dichogamy reduces within-flower selfing, while spontaneous or pollinator-mediated selfing within plant (i.e., geitonogamy) is more likely to occur (ROSSI *et al.* 2014). Seeds developed from self-pollination have lower viability and germination than cross-pollinated seeds (ROSSI *et al.* 2016).

Bumblebee observations and sampling

Field observations were carried out over a total of six days in two non-consecutive years (three days in both 2013 and 2015), between July 14th and July 23rd each year, during the flowering peak of *G. lutea* subsp. *symphyandra*. On each day we performed two observation periods of 45 min each, one in the morning and one in the afternoon between 11:00 AM and 4:00 PM, except for two days in 2015 when we could only perform one observation period because of bad weather. Observations were carried out by four trained operators in two fixed patches consisting of seven fertile stems each. For each stem, we recorded the number of whorls and the number of open flowers. During the observation periods, we recorded all bumblebee visits to open flowers and noted their type (i.e., cuckoo or free-living bumblebee), species and sex, number of flowers visited per whorl and plant, number of whorls visited per stem, number of stems visited per patch, and the time spent visiting flowers within each whorl. Females were easily identified by the presence of corbiculae and pollen actively collected on the hind tibia, and by other distinguishing features (e.g., shorter antennae, shape of last tergite). Species were identified in the field based on colour patterns and previous knowledge of the taxa present in the area. To further verify visual identifications at the species level, after each observation period we sampled by hand net or directly with plastic tubes bumblebees that visited plants of *G. lutea* in the surroundings of the focal patches. In addition, we collected all bumblebees that visited flowers for more than five minutes during the observation periods. Collected individuals were put in plastic tubes with ethyl acetate and brought to the laboratory for taxonomic identification by some of the present authors (LB, AF, GB, MG) with the help of dichotomous keys. Specimens are stored at the Laboratory of Plant Reproductive Ecology (Department of Biological, Geological, and Environmental Sciences) at the University of Bologna, Italy.

Data analysis

Since the focus of this article is not to describe temporal patterns of changes and because we did not find significant differences between years in a preliminary analysis, we pooled data from 2013 and 2015 in all analyses. Moreover, in a preliminary analysis we did not find significant differences between species in either the time spent on flowers or the number of flowers visited, therefore we pooled species data within cuckoo and free-living bumblebee types. We used Pearson's product-moment correlation coefficients to test for correlations between the number of flowers visited and the time spent visiting flowers within whorls and plants by bumblebees.

To test whether bumblebees showed differences in the time spent visiting flowers we used linear mixed models (LMMs). We used the log-transformed time (measured in seconds) spent foraging on flowers by individual bumblebees as response variable, and bumblebee type (i.e., males of cuckoo and free-living

bumblebees, workers of free-living bumblebees) as fixed effect. We tested two separate LMMs for the time spent within whorls and the time spent on the whole plant. In addition, we fitted two different LMMs including the number of whorls displayed by plants as additive factor or in interaction with bumblebee type to test for effects on the time spent on flowers at the whole-plant level. We included the observation interval, nested within day and year, as a random effect to account for temporal changes. In LMMs on the time spent on the whole plant, the best models in terms of goodness-of-fit were chosen based on the Akaike Information Criterion with correction for small sample sizes (AICc), selecting the model(s) with the lowest AICc value (BURNHAM & ANDERSON 2002). If ΔAICc values for some next best models were lower than 2, we used model averaging to calculate a weighted average of the parameter estimates (BURNHAM & ANDERSON 2002). After selecting the best models we performed pairwise comparisons between bumblebee types by estimating least-squares means with Tukey-adjusted p -values (LENTH 2020).

To evaluate differences in the number of flowers visited by bumblebees we fitted generalized linear mixed models (GLMMs) with Poisson error distribution and with the numbers of visited flowers as response variable, bumblebee type as fixed effect and observation intervals nested within day and year as random effects. We tested two separate GLMMs for the number of flowers visited within whorls and the number of flowers visited on the whole plant, and then performed pairwise comparisons between bumblebee types.

Analyses were performed and figures were drawn in R version 4.0.2 (R CORE TEAM 2019) using the R packages *ggplot2* (WICKHAM 2016), *lme4* (BATES *et al.* 2015), *emmeans* (LENTH 2020) and *MuMIn* (BARTON 2019).

Results

We observed a total of 350 bumblebees during 7.5 hours of observations over the two years of sampling, corresponding to three species of cuckoo bumblebees and four species of free-living bumblebees (Table 1). *Bombus rupestris* was the most abundant species among cuckoo bumblebees, while *B. terrestris* and *B. lapidarius* were the most abundant species among free-living bumblebees (Table 1). Overall, we only found cuckoo bumblebee males, and workers were more frequent than males in free-living bumblebees (ratio 1.6:1). We did not observe any gynes in either bumblebee type.

TABLE 1

Sample size of cuckoo and free-living bumblebee species observed throughout the study period. Cuckoo bumblebees do not have the worker caste, and no gynes were observed in either bumblebee type.

Species	Bumblebee type	Total	Workers	Males
<i>Bombus rupestris</i>	Cuckoo	91	–	91
<i>Bombus sylvestris</i>	Cuckoo	8	–	8
<i>Bombus vestalis</i>	Cuckoo	2	–	2
<i>Bombus terrestris</i>	Free-living	112	101	11
<i>Bombus lapidarius</i>	Free-living	94	17	77
<i>Bombus pratorum</i>	Free-living	41	33	8
<i>Bombus hortorum</i>	Free-living	2	2	0

The mean number (\pm SE) of whorls per plant was 4.93 ± 0.27 (min = 4, max = 6). On average, all bumblebees visited less than two plants per observation interval before leaving the flowering patches (cuckoo bumblebees = 1.45 ± 0.1 , free-living bumblebees = 1.48 ± 0.06 plants visited). We found significant positive correlations between the number of visited flowers and the time spent both within

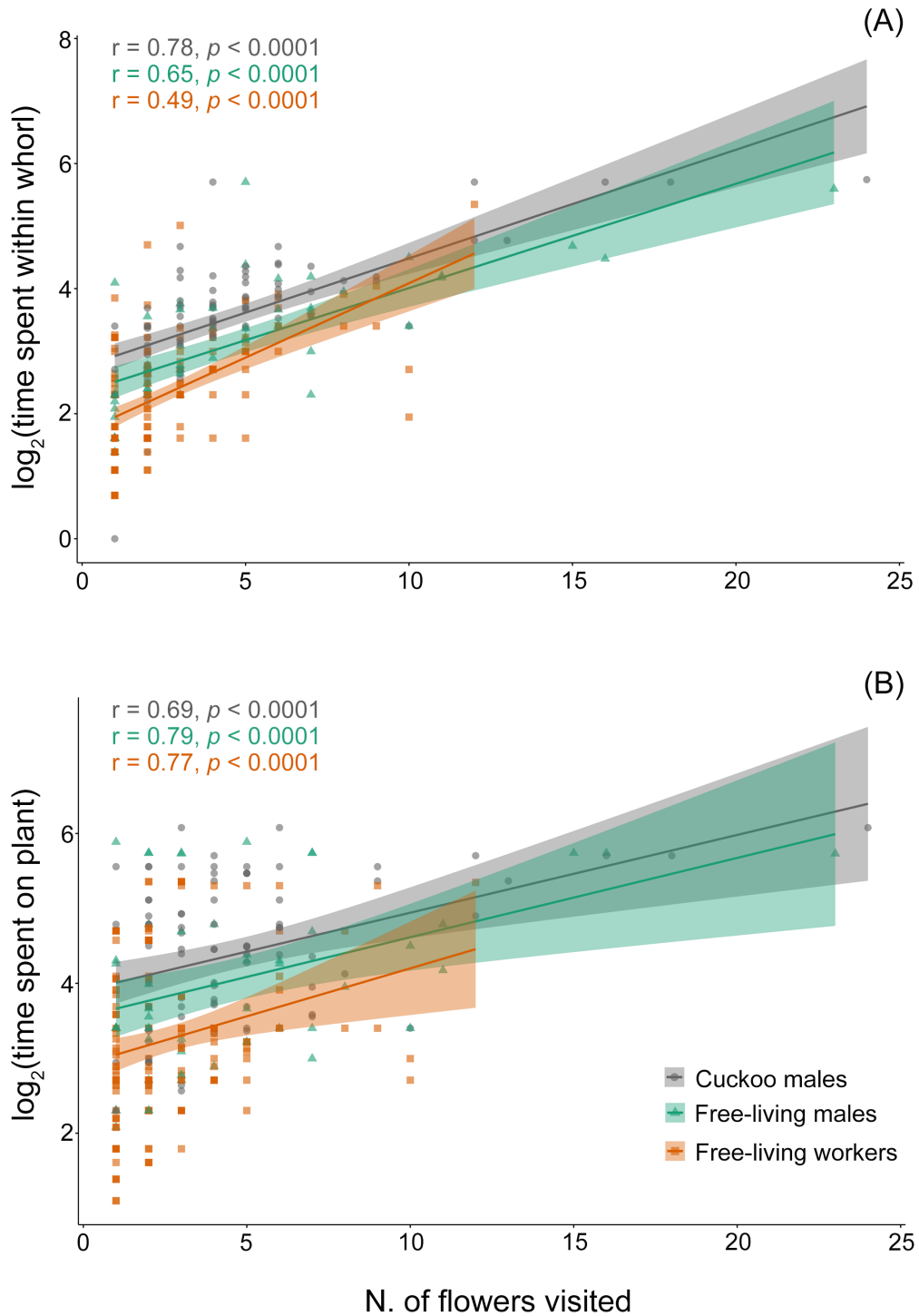


Figure 1 – Linear regressions and 95% confidence intervals between the number of visited flowers and the time (measured in seconds) spent (A) on flowers within whorl and (B) on the whole plant by cuckoo bumblebees and by free-living bumblebee males and workers. All cuckoo bumblebees were male.

whorls and on the whole plant. Within-whorl correlation was highest for cuckoo bumblebees, intermediate for free-living bumblebee males and lowest for free-living bumblebee workers (Fig. 1A). Within-plant correlation was lower for cuckoo bumblebees and comparably higher for free-living bumblebee males and workers (Fig. 1B).

Cuckoo bumblebees spent significantly more time visiting flowers within each whorl (106.2 ± 25.7 s) than both free-living bumblebee males (33.4 ± 5.2 s; Table S1, coefficient contrast = 0.87, $p < 0.0001$)

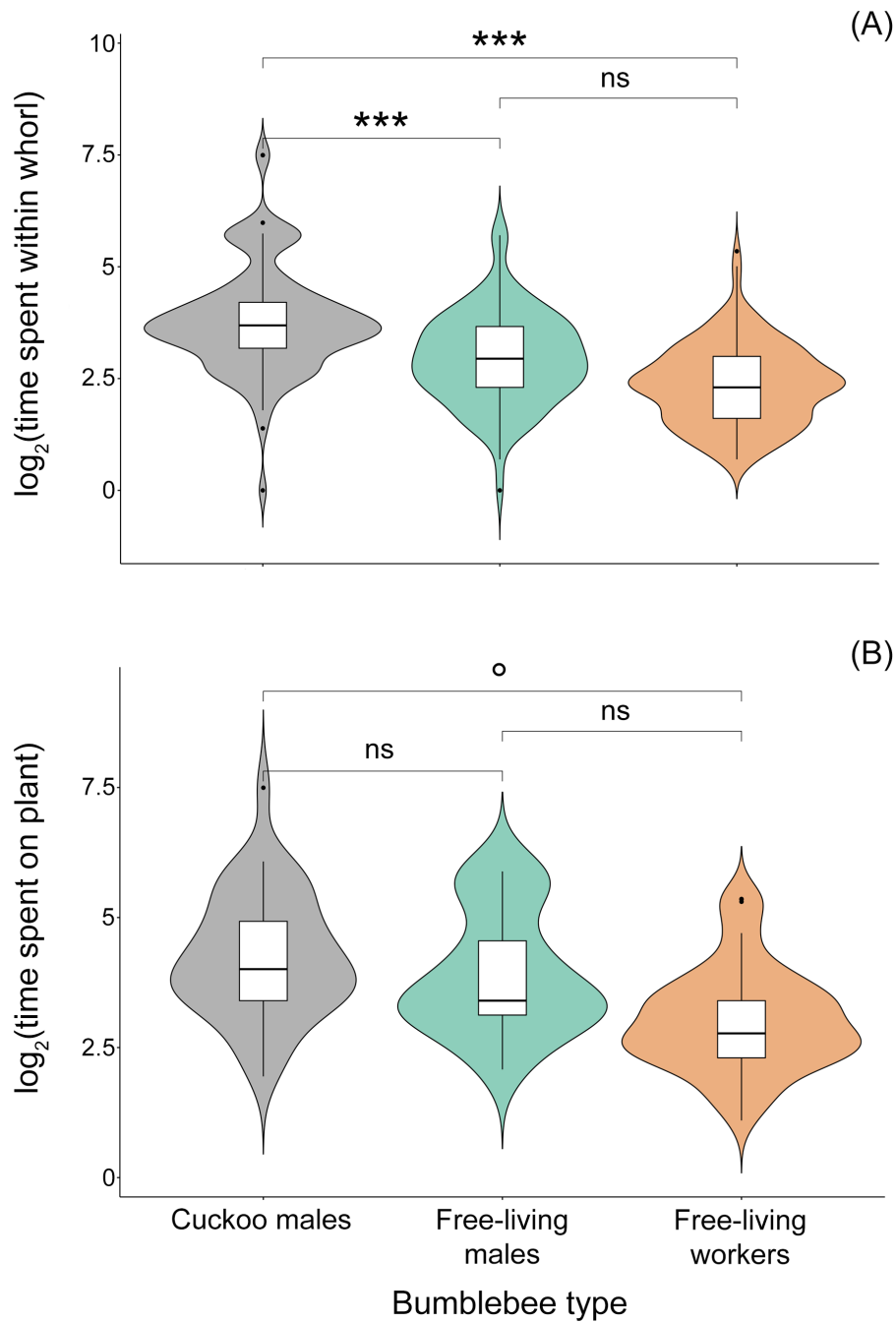


Figure 2 – Violin plots of time (measured in seconds) spent on flowers (A) within each whorl and (B) on the whole plant by bumblebee types (cuckoo bumblebee males, free-living bumblebee males and workers). All cuckoo bumblebees were males. $^{\circ}p = 0.059$; $***p \leq 0.0001$.

and workers (16.5 ± 1.9 s; Table S1, coefficient contrast = 1.20, $p < 0.0001$), which in turn did not significantly differ from each other (Fig. 2A, Table S1, coefficient contrast = 0.34, $p = 0.15$).

Cuckoo bumblebees spent almost significantly more time on flowers on the whole plant (153.1 ± 49.4 seconds) than free-living bumblebee workers (35.3 ± 6.9 s; Table S2, coefficient contrast = 0.69, $p = 0.059$) but not males (97.0 ± 24.4 s; Figure 2B, Table S2, coefficient contrast = 0.30, $p = 0.52$). Free-living bumblebee males and workers did not spend significantly different amounts of time visiting plants (Fig. 2B, Table S2, coefficient contrast = 0.39, $p = 0.43$). The number of whorls displayed by plants did not affect the flower visitation time of bumblebees (Tables S2–S3).

Cuckoo bumblebees visited significantly more flowers per whorl (4.74 ± 0.4) than free-living bumblebee workers (2.67 ± 0.2), but no more than males (4.73 ± 0.6), while free-living bumblebee males visited significantly more flowers than workers (Fig. S1A, Table S4). We did not find significant differences in the number of flowers visited at the whole plant level between bumblebee types (Fig. S1B, Table S5).

Tables S1–S5 and Fig. S1A–B can be found in Appendices 1 and 2, respectively.

Discussion

In this article we explored differences in the flower-visiting behaviour of cuckoo and free-living bumblebees. We provide the first empirical evidence of slower and significantly longer flower visits performed by cuckoo bumblebees compared to free-living bumblebees. We found that male cuckoo bumblebees visited more flowers and spent more time during each visit than both free-living bumblebee workers and males at the whorl level, but these differences were less marked at the plant level. Free-living bumblebee males visited more flowers than workers but not for significantly longer time periods.

Flower-visiting behaviour

The different behaviours observed between cuckoo bumblebee males and free-living bumblebee workers can be mainly related to different foraging needs. Bumblebee workers need to collect pollen for colony development, while at the same time keeping a positive balance between energy intake (through nectar) and the energy spent visiting flowers (HODGES & WOLF 1981; PYKE 1984). By contrast, cuckoo bumblebee males, as free-living males, do not collect pollen and visit flowers mainly for nectar. Consequently, the need to optimise energy intake over expenditure during foraging bouts is probably less important for cuckoo bumblebees than for free-living bumblebee workers.

Bumblebee workers visited fewer flowers for slightly shorter periods than free-living males. Though differences in visit duration were not statistically significant, mainly because of the high response variability of free-living bumblebee males, the different observed behaviours remain biologically relevant and support previous literature (OSTEVIK *et al.* 2010; WOLF & CHITTKA 2016). Shorter visits made by workers are likely driven by different foraging needs compared to males, as males do not collect pollen to sustain the colony similarly to cuckoo male bumblebees. Moreover, visits made by workers are usually driven by the quality and abundance of floral rewards, as workers tend to prioritize highly rewarding flowers to collect both pollen and nectar to optimize foraging bouts, while male visits are less constrained by reward availability (SMITH *et al.* 2019).

The longer visits and the higher number of flowers visited within whorls by cuckoo bumblebees cannot be attributed to different foraging requirements compared to free-living bumblebee males since neither of them provide pollen for the nest and mainly visit flowers for their own sustenance and to look for mates (GOULSON 2010; WOLF & MORITZ 2014). A possible explanation can be deduced from the slow

behaviour displayed by cuckoo bumblebees. Similarly to the only previous description of cuckoo foraging behaviour (PRYS-JONES & CORBET 1991), we frequently observed almost lethargic movements by cuckoo bumblebees during flower visits within whorls of *G. lutea*. We hypothesize that differences between cuckoo and free-living male bumblebees can be linked to different mating periods. Cuckoo bumblebee females tend to usurp free-living bumblebee nests at the beginning of colony development, when only the few workers of the first brood have been produced (KREUTER *et al.* 2012; LHOMME *et al.* 2013). Moreover, cuckoo bumblebees start to lay male and female eggs within 10 days from invasion (LHOMME *et al.* 2013), while free-living bumblebees start to lay male eggs between 30 and 40 days after the emergence of the first brood, and the last group of diploid eggs laid before male eggs usually develops into gynes (BOGO *et al.* 2018). However, the developmental time of cuckoo and free-living bumblebee sexual morphs (males and gynes) is comparable (KÜPPER & SCHWAMMBERGER 1995; BOGO *et al.* 2018). Consequently, a gap between the mating periods of cuckoo and free-living bumblebees can be expected. In our observations we only found cuckoo bumblebee males, suggesting that the peak flowering of *G. lutea* occurred when the mating period of cuckoo bumblebees was toward the end, therefore reducing the need for males to actively look for partners. By contrast, free-living bumblebees were likely still at the beginning of their mating period, compelling free-living male bumblebees to actively seek out partners and increase their mobility. In addition, cuckoo and free-living bumblebees may greatly differ in mating duration (e.g., 3 min versus more than 26 min, respectively; LHOMME *et al.* 2013), which can potentially affect male foraging behaviour, for example through distinct energetic requirements. Differences in the metabolism of cuckoo and free-living males may further emphasize behavioural differences. Further studies are needed to explore these possibilities.

Implications for pollination

Shorter visits made by worker bumblebees tend to favour constant foraging patterns and short-term floral specialization, which in turn can translate into a more efficient pollen dispersal between plants of a same species within a given population (SMITH *et al.* 2019). On the contrary, longer visits made by cuckoo bumblebees both within whorls and at the whole-plant level can likely increase the within-plant pollen flow (i.e., geitonogamy) and result in reduced plant fitness. Moreover, the higher number of flowers visited by cuckoo bumblebees, together with their sloppy foraging behaviour which increases the probability of touching receptive stigmas for longer time periods and with a larger body surface (personal observation AF, MG), suggests a further contribution to geitonogamous (i.e., between flowers on the same plant) pollen flow. The intermediate visitation time by male free-living bumblebees suggests that they may contribute intermediate pollination efficiency compared to cuckoos and workers, given their high potential for pollen transfer (OSTEVIK *et al.* 2010; WOLF & MORITZ 2014; OGILVIE & THOMSON 2015), while at the same time contributing to geitonogamous pollen transfer within plants of *G. lutea*.

Although we could not directly link bumblebee visits to plant fitness, we expect the longer flower visits of cuckoo bumblebees to reduce fruit set and seed set and germination. A previous study on the same population showed that self-pollinated flowers of *G. lutea* produced seeds with lower weight and germination rates compared to both open-pollinated and cross hand-pollinated flowers (ROSSI *et al.* 2016). The reduced fitness can be partially explained by inbreeding depression or by intrinsic genetic problems (e.g. founder effect or bottleneck) as a consequence of the isolation from other natural populations (ROSSI *et al.* 2016). However, the high abundance of cuckoo bumblebees observed in this study can act in addition to these factors and further reduce plant fitness in the study population. The transfer of pollen within plants facilitated by the longer visits of cuckoo bumblebees can increase geitonogamy and pollen discounting (BRUNET 2005), ultimately reducing plant fitness.

Among the 350 bumblebees observed in this study, almost a third (28.9%) were cuckoo bumblebee males. Because they are among the main pollinators of *G. lutea* (ROSSI *et al.* 2014), we expect them to play

an important role in the pollination success and ultimately on population persistence. Further analyses aimed directly at evaluating the effects of bumblebee visits on plant fitness would help understand the balance between positive (e.g., pollen flow) and negative (e.g., geitonogamy) contributions of cuckoos compared to free-living bumblebees. Given the widespread presence of cuckoo bumblebees, we expect that their visits can significantly affect the reproductive success of several plant species, and encourage future research to directly address this overlooked topic.

Data accessibility

Data and R scripts for analyses are available online: <https://doi.org/10.5281/zenodo.5161010>

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Conflict of interest disclosure

The authors of this preprint declare that they have no financial conflict of interest with the content of this article.

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