

MEANINGFUL WORDS IN CROWD NOISE: SEARCHING FOR VOLATILES RELEVANT TO CARPENTER BEES AMONG THE DIVERSE SCENT BLENDS OF BEE FLOWERS

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Abstract - Olfactory cues constitute one of the most important plant-pollinator communication channels. Specific chemical components can be associated with specific pollinator functional groups due to pollinator-mediated selection on flower volatile (FV) emission. Here, we used multivariate analyses of FV data to detect an association between FVs and the worldwide distributed pollinator group of the carpenter bees (*Xylocopa* spp.). We compiled FVs of 29 plant species: 9 pollinated by carpenter bees, 20 pollinated by other bee pollinator functional groups. We tested whether FV emission differed between these groups. To rule out any phylogenetic bias in our dataset, we tested FV emission for phylogenetic signal. Finally, using field assays, we tested the attractive function of two FVs found to be associated with carpenter bees. We found no significant multivariate difference between the two plant groups FVs. However, seven FVs (five apocarotenoid terpenoids, one long-chain alkane and one benzenoid) were significantly associated with carpenter bee pollination, thus being “predictor” compounds of pollination by this pollinator functional group. From those, β -ionone and (*E*)-methyl cinnamate presented the highest indicator values and had their behavioural function assessed in field assays. Phylogenetic signal for FVs emission was weak, suggesting that their emission could result from pollinator-mediated selection. In field assays, the apocarotenoid β -ionone attracted carpenter bees, but also bees from other functional groups. The benzenoid (*E*)-methyl cinnamate did not attract significant numbers of pollinators. Thus, β -ionone functions as a non-specific bee attractant, while apocarotenoid FVs emerge as consistent indicators of pollination by large food-foraging bees among bee-pollinated flowers.

Key Words - Floral VOC, β -ionone, (*E*)-methyl cinnamate, solitary bee, *Xylocopa*.

INTRODUCTION

About 87.5% of flowering plant species depend on animal pollination for their reproduction at some level (Ollerton et al. 2011). Hence, pollinators that are more effective or that are present in greater abundance can exert significant selective pressures towards floral traits of their preference in a process known as pollinator-mediated selection (Schiestl and Johnson 2013). Pollinator-mediated selection of floral signals is often mediated by animal perceptual abilities and behaviour (Schiestl 2017; Schiestl and Dötterl 2012). This can result in convergence of characters in flowers that are not closely related in their phylogeny yet share the same pollinator (Fenster et al. 2004; Kantsa et al. 2017). As convergent traits often indicate pollinator-mediated selection, there is a substantial interest in understanding how different floral traits relate to the sensorial abilities of their pollinators (Schiestl and Johnson 2013).

Plant-pollinator communication can happen through several channels, among which olfactory stimuli stand out as one of the most important (Kessler et al. 2008; Raguso 2004). Knowingly, plants use flower volatiles (FVs) for attracting their animal pollinators to flowers, besides eliciting a series of other behaviours like courtship, landing, feeding and oviposition (Dobson 1994). Although fragrant flowers emit bouquets containing from a few to more than a hundred different FVs, specific chemicals can be associated with specific pollinator groups. For instance, bat-pollinated flowers of different plant families emit sulphur-containing FVs (Dobson 2006). Bee-pollinated oil-flowers usually emit diacetin, a volatile that attracts a relatively narrow range of oil-collecting bees (Schäffler et al. 2015). However, little is known if specific FVs are associated with other important pollinator groups, such as the cosmopolitan group of solitary large-sized bees, the carpenter bees of the genus *Xylocopa*. These carpenter bees have a worldwide distribution from tropical and subtropical to temperate regions of the planet, with some species endemic to islands and others found even in Nearctic regions. Despite being a cosmopolitan and diverse taxon, *Xylocopa* bees bear a combination of traits that distinguish their natural history and possibly their role as pollinators from other bees (Leys et al. 2002). As their most distinguishable traits, *Xylocopa* carpenter bees present extremely strong mouthparts used to dig into wood or soil to build their nest cavities in addition to a stiff blade-like mouthpart used to pierce some of the flowers they visit for food (Michener 2007). In general, we can expect *Xylocopa* and other large-sized solitary bees to be effective pollinators of both native plants and crops. This is likely due to their longer flight distances, traplining behaviour

(Janzen 1971), ability to perform buzz-pollination and physical strength to open and access certain specialized flower morphologies (Córdoba and Cocucci 2011; Stephanie et al. 2015). These features may represent attributes that make them more effective in transferring pollen when compared to other bees foraging for pollen and nectar in a context of diverse pollinator communities. Specifically, carpenter bees are the sole pollinators of several plant species, mainly orchids from the Palaearctic, Afrotropical and Neotropical regions (Wappler et al. 2015). However, they can share their pollination role with other large bees in a myriad of more generalist plants (Kearse 2010). In some regions where other common groups of large bees are not present, like in the case of bumblebees in sub-Saharan Africa, carpenter bees may assume the ecological role of the main pollinators of robust and complex flowers (Wappler et al. 2015). On the other hand, some carpenter bees also show a remarkable behaviour of nectar robbery, that can reach 100% of the visits in some plant species, but that can also result in pollination in other cases (Bronstein et al. 2017; Kearse 2010). Consequently, pollinator-mediated selection could favour specific FVs acting either as preferential attractants of carpenter bee pollinators or as chemical deterrents of nectar-robbing by them. Previous work on a small group of closely related co-flowering plants exposed to the same pollinator community showed significant differentiation of the floral scents of plants exclusively pollinated by carpenter bees (Nunes et al. 2017). Thus, finding chemicals relevant to the interaction with a specific group of bee pollinators in a broader context proved to be an ambitious but achievable challenge, in view of the overwhelming diversity and complexity of floral scent blends of bee-pollinated flowers (Knudsen et al. 2006).

Although there have been studies involving the ability of other bees like honeybees and bumblebees in differentiating distinct FV mixtures in quality and quantity (Laloi and Pham-Delègue 2004; Paldi et al. 2003), little is known about which are the FVs relevant to carpenter bees. In this context, a systematic comparison across diverse plant species may reveal which FV blends are associated to the functional group of pollinators represented by carpenter bees. The following step would be to assess what types of behaviour the associated FVs may elicit. Here, we compared FV composition across a compilation of plant species in two categories: plants pollinated mainly by carpenter bees and plants pollinated by bee genera representing other pollinator functional groups. Further, we tested for a phylogenetic signal on FV emission to exclude the hypothesis that any of the observed emission patterns were due to shared

phylogenetic history. This approach revealed seven FVs specifically associated with carpenter bees, from which two had their behavioural effect assessed in field assays.

METHODS AND MATERIALS

Data Compilation. Data on pollinators and FV profiles were compiled through bibliographical research in Google Scholar platform. We used the keywords “*Xylocopa pollinat**” or “*carpenter bee pollinat**” to search for plants pollinated by carpenter bees in the literature. For plants pollinated by other bee genera, we first found plants with FV profiles described and then searched for pollinators using the name of the plant species plus “*pollinat**”. The information on the composition of FVs of the selected species was mainly gathered from the semiochemical database Pherobase (El-Sayed 2020) and their respective volatiles constitution and constituent percentage of each volatile were detailed according to the reference articles listed for each plant on this platform. To search for FV profiles that were not in Pherobase, we used the name of the plant species combined with the keywords “*floral volatiles* or *bouquet* or *blend* or *odour* or *perfume*”. The FVs were categorized into main classes based on the review of diversity and distribution of floral aromas compiled by Knudsen, Eriksson, Gershenzon, & Ståhl (2006). To avoid any errors due to the existence of synonyms to refer to a given FV, we used the number of registry on CAS (Chemical Abstracts Service of the Chemical American Society), which is unique to each chemical compound (Morgan 1965), to organise the FV list and check for duplicates.

In order to be included in this work, plants pollinated by *Xylocopa* bees should have been reported in the literature or in this paper as mainly pollinated or with more than one third of the legitimate flower visits performed by *Xylocopa* spp. Also, their FVs should have been described either in the same paper or in other paper from literature. We strictly selected plant species proven to be pollinated, not only visited, by *Xylocopa* spp. as we were looking for floral compounds positively selected by these bees in the flowers scent blends. We ended up with nine plants species, eight with FVs characterized in literature and one with FVs sampled by us (see below). Despite the existence of relatively small-sized *Xylocopa* species, all the species of this genus included in this work were at least 15 mm in length. Thus, this allowed us to classify them as belonging to the functional group represented by large short-tongued bee pollinators (Hoehn et al. 2008).

For the second group of species, we selected plants identified as pollinated by bees from genera other than *Xylocopa* (hereafter ‘pollination by other bees’), which forage for pollen or nectar. Plants exclusively pollinated by male *Euglossini* bees were not included, since males of this group are known to visit certain flowers to collect their perfumes, being attracted by very specific FVs (Lunau 1992). This second group of plants could potentially be much larger than the first, hampering our ability to make meaningful comparisons. Therefore, we included in this work 20 species found in the literature with both information about main bee pollinator and composition of FVs. Because of the significantly small number of plant species with FVs described, we first compiled those species with FVs already described and then searched for their main pollinators, with special attention to gather a group of plant species from different families and pollinated by bees from different genera.

Our dataset of plant-pollinator interactions included mainly interactions studied in the native geographic range of the plant species (22/29), which thereby would have a shared evolutionary history with the local pollinator fauna. However, cultivated plants studied out of their native range were also included (7/29) in both the group of plants pollinated by carpenter bees (1/29) and the group of plants pollinated by other bees (6/29, Online Resource 1).

Collection of FVs. Additional unpublished data of the floral scent of the orchid *Cattleya loddigesii*, a species that was opportunistically observed being pollinated mainly by *Xylocopa* bees (E. Parra, unpublished data), was collected in the greenhouse using solid phase micro-extraction (SPME) and analysed at the laboratory using gas chromatography coupled to mass spectrometry (GC-MS) by the authors. This extra data point increases the number of data points in the dataset and makes public a novel orchid-pollinator interaction. We used three flowering individuals collected in the field at the municipality of São Luiz do Paraitinga, São Paulo, Brazil, and kept in the University’s greenhouse. Open flowers, inflorescences or parts of them were wrapped in polyester bags (27 × 41 cm) and left for one to three hours to concentrate FVs and reach flower-air equilibrium. Thereafter, bags were perforated with a pin and their FVs containing air were exposed to a solid phase micro-extraction (SPME) syringe with a polydimethylsiloxane fibre (PDMS, 100 µm, Supelco, Bellefonte, PA) for 15 min. This procedure was performed on sunny and partially cloudy days at 20-30°C at the same

daytime that fragrances were most often detected by human smell sense under natural conditions in the field (between 10 am to 13 pm).

Immediately after collection, SPME fibre samples were directly injected into a gas chromatograph (2010A, Shimadzu, Tokyo, Japan) coupled to a quadrupole mass spectrometer (QP2010, Shimadzu) using a DB5 capillary column (30 m length, 0.32 mm internal diameter and 0.25 μ m film thickness, J&W Scientific, Folsom, CA, USA) with helium as a carrier gas (flow of 1 mL.min⁻¹). Injection was performed in splitless mode, and the fibre was kept for 20 min in the injector at 200°C with transfer line at 240°C to elute FVs. The oven temperature started at 50°C and then increased by 10°C min⁻¹ to a maximum temperature of 250°C and was then held for 10 min until the end of the run. Mass spectra were recorded by electron impact (EI) at 70 eV using the SIM mode. Compound peaks were individually integrated and had their Kovats Retention Index (RI) calculated from a previously injected homologous series of n-alkanes (C8-C20) using the data acquisition software GCMSsolution (Shimadzu, Tokyo, Japan). Finally, each compound peak was identified by comparison of both mass spectrum and RIs to those of the NIST05 and NIST online library (Linstrom and Mallard 2011) and The Pherobase semiochemical database (El-Sayed 2020).

Multivariate Analysis of the FVs Data. We created a matrix with all plant species (pollinated by carpenter bees and pollinated by other bees) and their respective FVs in relative amount (%) averaged per plant species when the work describing the floral scent presented results for more than one sample (Online Resource 2). In spite of the fact that absolute amounts of FV could be a more comparable measure of volatile emission across different plants, we opted for using the relative amounts as this measure is available in most publications on floral scent blends, while the absolute amounts are missing from some of the literature. Each entry represents the average relative percentage of a given FV on the scent of a given species. To allow the multivariate analysis to include all FVs listed in literature, we converted the so called “trace” amounts of FVs from papers to 0.001% in our dataset. This “species \times FVs” matrix of floral scents did not meet the assumption of multivariate homogeneity of group dispersions (*ANOVA*, $F_{1,28} = 1.3718$, $P > 0.05$, performed with *vegan* R-package, Oksanen et al. 2016) and the assumption of multivariate normality of variances (*Shapiro–Wilk test*, $W = 0.033815$, $P < 0.001$, performed with *mvnrmtest* R-package, Jarek 2012). Thus, we used a non-parametric approach in our multivariate analysis.

We applied the Hellinger transformation to make the floral scent data containing many zeros (e.g., compounds completely absent in certain species, but present in others) suitable for multivariate analysis (Legendre and Gallagher 2001). A non-parametric multiple response permutation procedure (MRPP) with the average Bray–Curtis distance among samples weighted to group size and 999 permutations assigning the observed relative amounts of FVs in % at random to the different plant species was conducted to test differences in floral scents between plants pollinated by carpenter bees and plants pollinated by other bees (Mielke and Berry 2007). The MRPP test was performed with the *vegan* R-package.

To detect specific floral scent compounds associated with any of the two group of the plant species, we performed an indicator compound analysis (ICA) with 999 random permutations. The computed indicator value (IV) of each compound reflects both its relative abundance (specificity – ‘A’, the probability that a species belongs to the target group of species, given that the compound has been found in it) and its relative frequency (fidelity – ‘B’, the probability of finding the compound when the species belongs to the target groups of species). The associated P-values determined whether specific compounds are significant indicators of a certain groups of species (De Caceres and Legendre 2009; Dufrêne and Legendre 1997). The ICA was performed with the *indicespecies* R-package (De Caceres and Legendre 2009).

To characterize floral scent similarities across the whole scent profile among the plant species, we used the non-metrical multidimensional scaling (NMDS) ordination on a matrix of Bray-Curtis distance on the relative proportions of odour compounds (in % of the total blend). For a better visualization of the ordination, we excluded data from the plant *Cucumis melo* as it did not share any of its floral volatiles with any of the other plant species studied, being always completely dissimilar from any other, thereby adding no information to an ordination based on relative dissimilarities. The NMDS ordination was performed using the metaMDS function ($k = 5$ dimensions and maximum of 100 random starts) and the vectors of maximum correlation between the NMDS scores and relative abundances of the seven floral volatiles found to be indicative of pollination by carpenter bees were calculated using envfit function, both in the *vegan* R- package (Oksanen et al. 2016).

Phylogenetic Signal of Floral Volatile Emission. We built a phylogenetic hypothesis representing evolutionary relationships among all species following the consensus

supertree of Zanne et al. (2014). The divergence times for major Angiosperm lineages used followed Bell, Soltis and Soltis (2010). With this tree, we obtained phylogenetic distances using the cophenetic function of *ape* R-package (Paradis and Schliep 2019).

We conducted a Mantel test between the matrix of floral volatiles and the matrix of phylogenetic distances to assess phylogenetic signal of floral scent among the 29 species. We assessed the phylogenetic signal of the specific compounds that were found to be indicators of the carpenter bee group with the *K* statistic using *phytools* R-package (Blomberg et al. 2003; Revell 2012). It analyses the amount of variation in one trait among species that is correlated with the phylogenetic distances under the expectation of Brownian motion evolution. Values of $K > 1$ indicate that related species are more similar than expected (Blomberg et al. 2003). The observed *K* for the indicator compounds was compared with a null distribution generated by 10,000 random trees created by mixing species into the null phylogenies to analyse its significance. Values of *K* significantly different from 0 indicate the existence of some level of phylogenetic signal.

Assays. We performed assays in urban and semi-urban areas with the two FVs found to be associated with carpenter bees: the apocarotenoid monoterpene β -ionone and the benzenoid (*E*)-methyl cinnamate. These two FVs were chosen as they presented the first two highest indicator values in the ICA. The assays were performed from December 2018 to April 2019 and complemented in January 2020, in green areas at the University Campus and in suburban areas in the surroundings. The vegetation is composed of house gardens and remains of semideciduous woodland of the Atlantic forest domain (Velooso et al. 1991). The pollinator community in the sites of assays is composed by diverse bee groups, with the dominance of medium to small-sized social bees, including invasive Africanized honeybees (Agostini and Sazima 2003).

Specifically, we aimed to test (1) if carpenter bees are attracted by each of these two FVs presented individually as well as (2) if carpenter bees prefer one compound over another when presented in the same assay. As our results showed that these two FVs are found in distinct plant species, we exposed each FV in separate baits. We conducted three types of assays: (1a) two-choice assays with β -ionone vs. control, (1b) two-choice assays with (*E*)-methyl cinnamate vs. control, and (2) multiple-choice assays with β -ionone, (*E*)-methyl cinnamate and control baits exposed simultaneously.

The assays were performed from 6:20 to 13:00 h on non-rainy days. Each replicate consisted of a pair of circular filter-paper baits (Whatman #1; 11 cm diameter) hung by a cotton line on tree trunks or bushes of the gardens respecting the distance of 1 m within each lure or control bait. In each pair, 0.5 mL of pure β -ionone or (*E*)-methyl cinnamate analytical standards (Merck, São Paulo, Brazil, >90% purity) was applied to the lure paper, and nothing was applied to the control paper. As (*E*)-methyl cinnamate has its melting point at 34-38° C, we used a warm bath to make it liquid prior to application on the lure paper. In each daily trial, a group of three to seven lure-control pairs or trios was continuously exposed and observed in the field for 1 to 4.25 hours, totalling an effort of 63.27 scented baits times hours of exposure (hereafter, baits.hours) for β -ionone vs. control (1a), 66 baits.hours for (*E*)-methyl cinnamate vs. control (1b), and 145.02 baits.hours for multiple-choice assays (2), being 72.51 baits.hours for each of the two FVs tested together. Each of these three categories of assays was performed at two to five different sites distant at least 1 km from each other. A choice was recorded each time an insect touched or approached a lure or control paper to a distance of at least 10 cm. All insects that visited the papers were recorded and immediately identified to the genus level when possible. To avoid pseudoreplication of the insect visits to the paper baits, we temporarily hold the insect visitors in vials when possible and only accounted for visits of insect that could be clearly differentiated one from another during the visits due to differences in body size or morphology. When identification in situ was not possible, a specimen was collected and stored for later identification. We then tested preference between treatments using the exact binomial test of goodness-of-fit for the two-choice assays with the function `binom.test` or the randomization test of goodness-of-fit using 10,000 Monte-Carlo simulations for multiple-choice tests in *xnomial* R-package (R Development Core Team 2020). As we were interested in testing the attraction of the specific FVs to carpenter bees in comparison to other pollinator functional groups, we performed separated tests for functional group (carpenter bees and other food foraging bees). Finally, to specifically test if a selected FV attracted more pollinators when exposed alone than when exposed together with other FV, we performed a simple Wilcoxon test comparing the overall number of pollinators per hour per scented bait attracted in two-choice assays with those in multiple-choice assays, considering the assay as the sampling unit.

RESULTS

We retrieved 348 compounds identified among the FV samples collected in vivo and in the literature from the 29 species of plants compiled. They could be categorized as fatty acid derivatives (122), benzenoids (80), monoterpenes (61), sesquiterpenes (33), irregular terpenes (18), nitrogen containing compounds (8), miscellaneous cyclic compounds (7), sulphur containing compounds (7), C5-branched chain compounds (2) and not identified (10) (Online Resource 2).

The MRPP did not indicate an overall multivariate difference between the floral scents (relative percentages) of carpenter bee-pollinated and other bee-pollinated plant species (*MRPP*, $A = 0.003667$, $\delta_{observed} = 70.93$, $\delta_{expected} = 71.19$, $P > 0.05$). Convergently, the NMDS analysis ($stress = 0.086$; two convergent solutions found after 20 trials) did not evidence any clear separation between plants pollinated by carpenter bees and plants pollinated by other bees based on their FVs profiles (Figure 1). However, the Indicator Compound Analysis indicated seven FVs to be significantly associated with plants pollinated by carpenter bees: (*E*)-nerolidol, geranial, geranylacetone, neral, tetradecane, β -ionone and (*E*)-methyl cinnamate. From those seven FVs, β -ionone and (*E*)-methyl cinnamate presented the two highest indicator values (Table 1).

There was no correlation between the matrix of floral volatiles from the 29 plant species and its phylogenetic distances, suggesting no phylogenetic signal for FV emission profile (*Mantel test*, $r = 0.022$; $P > 0.05$). The presence of β -ionone and of (*E*)-methyl cinnamate in the floral scent showed no phylogenetic signal, with K values not different from 0 ($K = 0.435$, $P > 0.05$ and $K = 0.510$, $P > 0.05$, respectively), suggesting that closely related species are less similar than expected.

In the two-choice assays, β -ionone attracted a significant number of carpenter bees (14 visits to baits out of 15 visits, *exact binomial test*, $P < 0.001$), but also a significant number of other bees from other functional groups (*Trigona spinipes* stingless bees, nine choices to baits out of nine total visits, *exact binomial test*, $P = 0.004$) and higher number of male euglossine bees (98 visits to baits out of 98 visits, *exact binomial test*, $P < 0.001$). All carpenter bees made relatively short visits (less than five seconds), never landing on the baits. Similarly, *Trigona* stingless bee workers never landed on the baits, but eventually spent more time hovering around a bait. Male euglossines usually spent more time on the scented baits, landing on them and performing their stereotypical perfume-collection behaviour (Eltz et al. 2005; Vogel 1966). (*E*)-methyl cinnamate did not attract any pollinators in numbers high enough

(always lower than five visits) to allow statistical inference based on the number of choices of lures against controls in the two-choice assays (Figure 2, a and b).

In the multiple-choice assays, β -ionone also attracted significant numbers of carpenter bees ($P < 0.01$, 10,000 simulations) and male euglossines ($P < 0.001$; 10,000 simulations), while (*E*)-methyl cinnamate did not attract a number of pollinators sufficient for statistical inference (Figure 2c). Interestingly, β -ionone attracted greater numbers of pollinators in two-choice assays than in multiple-choice assays (*Wilcoxon test*, $V = 91$, $P = 0.002$). While β -ionone baits tested alone against controls yielded 3.2 ± 3.4 visits per bait per hour ($n = 7$ assays), β -ionone baits tested in multiple-choice bioassays yielded 0.6 ± 0.3 visits per bait per hour ($n = 6$ assays).

DISCUSSION

We have not detected significant multivariate differences between floral scent blends of plants pollinated by carpenter bees and plants pollinated by other bees. Yet, the results of Indicator Compound Analysis showed that, out of 125 chemicals compiled for plants pollinated by carpenter bees, seven were associated with flowers pollinated by these large solitary bees, either by their high relative abundance or by high relative frequency among carpenter bee-pollinated flowers (Table 1 and Online Resource 2). From those seven FVs, β -ionone and (*E*)-methyl cinnamate presented the two highest indicator values, being “indicator” compounds of pollination by carpenter bees. This supports the hypothesis that flowers pollinated by a specific functional group differ in some recognizable floral volatiles, despite the wide diversity of scents among bee-pollinated flowers.

For most Angiosperms, floral scent composition tends to be strongly species-specific (Azuma et al. 1997; Barkman et al. 1997). This fact may have led to weak phylogenetic signal of floral scent constitution found for all 29 species. Knudsen et al. (2006) did not find phylogenetic clusters nor detectable patterns among floral blends across the Angiosperms, and together with our results, it shows the lack of reliability of the floral perfume chemicals to be used as a surrogate of phylogenetic relatedness, due to their great evolutionary lability (Barkman 2001; Williams and Whitten 1999). Similarly, community-wide studies also failed to detect phylogenetic signal on FV composition (Filella et al. 2013; Gervasi and Schiestl 2017; Kantsa et al. 2017). It is not rare to encounter floral blends composed by many biosynthetically closely related

compounds, especially in terpenoid compounds (Gershenzon and Kreis 1999). Additionally, there are some chemical compounds that may function neither as attractant nor as repellent, but instead they would modify these functions of other compounds of the floral blend (Kessler et al. 2013; Williams and Whitten 1983). Nevertheless, dissimilarities in floral fragrances may not necessarily be adaptive, remaining in populations as a result of genetic drift or phenotypic plasticity (Ackerman et al. 1997; Olesen and Knudsen 1994). Thus, as we used plant species from different biogeographic regions, climates and ecosystems, we hypothesize that abiotic (e.g. air temperature and moisture) and ecological factors (e.g. level of pollinator specialization) may also play important roles in explaining floral scent variation in the broad context of bee pollination (Kantsa et al. 2017; Majetic et al. 2009).

In our field assays, the irregular terpene β -ionone acted as an effective attractant of carpenter bees. However, the attractiveness of this single volatile is not specific as β -ionone also attracted social stingless bees and male euglossines. The frequency of visits by male euglossines to β -ionone were up to seven-fold the frequency of visits of *Xylocopa* carpenter bees (Figure 2, b and c). This discrepancy might be because those male euglossines actively collect and use β -ionone to compose their pheromones (Eltz et al. 2005, 2006). Thus, in the case of perfume collection, FVs act both as attractants and rewards and we expect higher numbers of these insects in the lures of their interest. In fact, both β -ionone and (*E*)-methyl cinnamate are known to attract perfume-collecting males of various euglossine bee species (Eltz et al. 2006; Nemésio 2009; Schiestl and Roubik 2003). Therefore, this work expands our knowledge on plant-pollinator communication by including both carpenter bees (*Xylocopa*) and stingless bees (specifically *Trigona* sp., Meliponini) in the role of bee groups attracted by β -ionone (El-Sayed 2020). Noteworthy, studies on the floral visitors and pollination mechanisms of plants pollinated by carpenter bees have rarely accounted for exclusive attraction to those bees: in the cases compiled in this study, only two orchid species were exclusively visited by carpenter bees (Braga 1977; Matias et al. 1996). Thereby, in general, exclusive pollination by carpenter bees may not be reached solely by the emission of specific scent blends, but instead by a combination of volatiles and morphological traits that would exclude other functional groups as pollinators (Córdoba and Cocucci 2011; Ellis and Johnson 2009; Nunes et al. 2017). Indeed, some of the flowers compiled in this study present morphologies that make it much less likely that small bees act as pollinators (Figure 3) (Junker and Parachnowitsch 2015).

Our work shows a significant relationship of compounds derived from carotenoid pigments, i.e. apocarotenoids, with pollination by large-bodied bees such as *Xylocopa* carpenter bees. Five out of seven compounds found to be significant indicators of pollination by *Xylocopa* are apocarotenoids, namely (*E*)-nerolidol, geranial, geranylacetone, neral, and β -ionone (Table 1). Moreover, in our survey in field conditions with one benzenoid ((*E*)-methyl cinnamate) and one apocarotenoid (β -ionone), only the apocarotenoid effectively attracted *Xylocopa* carpenter bees (Figure 2). Remarkably, the carotenoid-pigmented flowers of the Amaryllidaceae *Narcissus cuatrecasasii* elicit relatively large amounts of β -ionone and are pollinated by large-bodied *Anthophora spp.* bees (Dobson 2006; Pérez-Barrales et al. 2006). Additionally, three orchids included in our dataset (*Caularthron bicornutum*, *Constantia cipoensis* and *Zygopetalum crinitum*) are pollinated by deceit by *Xylocopa* and may rely on emission of relatively large amounts of apocarotenoids FVs to lure bees into visiting their flowers (Table 1, Online Resource 2). These facts together with the significant association of five apocarotenoids with the group of species mainly pollinated by *Xylocopa* in our dataset allow us to hypothesize that volatile apocarotenoids are specifically connected to pollination by large bees foraging for nectar and pollen in the chemically diverse context of bee flowers, not only to pollination by specific perfume-foraging male euglossines bees. Further research should thus investigate why emission of apocarotenoid volatiles among bee-pollinated flowers would be specifically associated to large-bodied bees while also being used as chemical cues by bees in general, not only by large bees (Dudareva et al. 2006). Would apocarotenoid emission on flowers be a result of selection by these long-distance travelling pollinators on the plants they visit? Would apocarotenoid emission on flowers emerge from other flower traits associated to pollination by large bees, such as relatively large amounts of yellow pigments in the flowers?

In addition to always being capable to perform buzz-pollination, large bee pollinators can travel long distances, transport higher loads of pollen and have increased foraging capacity in lower temperatures, which can make of them more effective pollinators in comparison to small bees (De Luca and Vallejo-Marín 2013; Stone 1994). Importantly, four out of the nine plant species pollinated by carpenter bees included in our dataset are cultivated for food (cowpea, *Vigna unguiculata*; moringa tree, *Moringa oleifera*; passionfruit, *Passiflora edulis*, and eggplant *Solanum melongena*). Thus,

additional emission of β -ionone at these crops could increase attraction of carpenter bee pollinators and, consequently, increase yields (Yamamoto et al. 2012).

Curiously, the reduced attraction of β -ionone to pollinators when tested together with (*E*)-methyl cinnamate in our multiple-choice assays evidences a possible conflict of functions between different chemicals emitted together (Figure 2, b and c). Such conflict may have consequences to the attraction and behaviour of pollinators in nature and eventually determine the level of attractiveness of complex scent blends to specific pollinators. Specific volatiles may act dually as attractants for mutualists while repelling antagonists, or even filter out ineffective pollinators among the range of possible visitors (Junker and Blüthgen 2008, 2010; Laloi et al. 2000). Lunau, Papiorek, Eltz, and Sazima (2011) showed that avoidance of some floral traits by a group of pollinators can provide another group of pollinators that do not show preferences with a private niche to explore. Thus, perception and behavioural preferences of carpenter bees to β -ionone and (*E*)-methyl cinnamate need to be further explored through other types of assays, e.g. proboscis extension response (PER) and Electroantennogram studies.

In summary, we show that in the context of bee-pollination, plants from distinct lineages rely on emission of β -ionone and possibly other apocarotenoid volatiles to attract their carpenter bee pollinators. Future research on the attractiveness of β -ionone and (*E*)-methyl cinnamate attractiveness in ecological contexts other than the one in this study and on the functions of the other five FVs found here to be associated with carpenter bees may considerably expand our knowledge of plant-bee communication.

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644

645 **Table 1** FLORAL VOLATILE ORGANIC COMPOUNDS (FVS) SIGNIFICANTLY ASSOCIATED WITH CARPENTER BEES AND
646 PLANT SPECIES THAT EMIT THEM, NINE OF THEM POLLINATED BY CARPENTER BEES (IN BOLD). THE TWO VOLATILES
647 ASSOCIATED WITH CARPENTER BEES WITH THE TWO HIGHEST INDICATOR VALUES IN THE INDICATOR COMPOUND
648 ANALYSIS (SINGLE ASTERISKS) WERE SELECTED TO BE TESTED FOR THEIR BEHAVIOURAL EFFECT ON DIURNAL
649 POLLINATORS IN FIELD ASSAYS. THE COMPLETE LIST OF PLANTS AND VOLATILES COMPILED IN THIS WORK CAN BE
650 FOUND IN THE ONLINE RESOURCE 2

	FVs	(E)-nerolidol	geranial	geranylacetone	neral	tetradecane	β-ionone*	(E)-methyl cinnamate*
	Indicator values	0.577	0.577	0.577	0.577	0.557	0.667	0.745
	P values	0.041	0.023	0.021	0.023	0.037	0.006	0.003
Plant families	Plant species	Average relative abundance %						
Actinidiaceae	<i>Actinidia chinensis</i>	-	-	0.17	-	0.34	1.2	-
Fabaceae	<i>Vigna unguiculata</i>	-	-	-	-	-	-	5.22
Lecythidaceae	<i>Couroupita guianensis</i>	-	1.6	-	1.7	-	-	-
Moringaceae	<i>Moringa oleifera</i>	13.4	-	-	-	-	-	-
Orchidaceae	<i>Cattleya loddigesii</i>	-	-	-	-	4.64	-	-
	<i>Caularthron bicornutum</i>	-	-	16.9	-	-	8.6	1
	<i>Constantia cipoensis</i>	-	1.5	8	<0.1	-	1	3
	<i>Zygopetalum crinitum</i>	12.2	3.3	-	<0.1	-	-	<0.1
	<i>Zygopetalum mackayi</i>	-	-	-	-	1.17	-	-
Passifloraceae	<i>Passiflora edulis</i>	-	-	-	-	2.1	-	5.9
Rutaceae	<i>Murraya paniculata</i>	0.1	-	-	-	-	-	-
Solanaceae	<i>Solanum melongena</i>	-	-	10.09	-	2.41	3.16	-

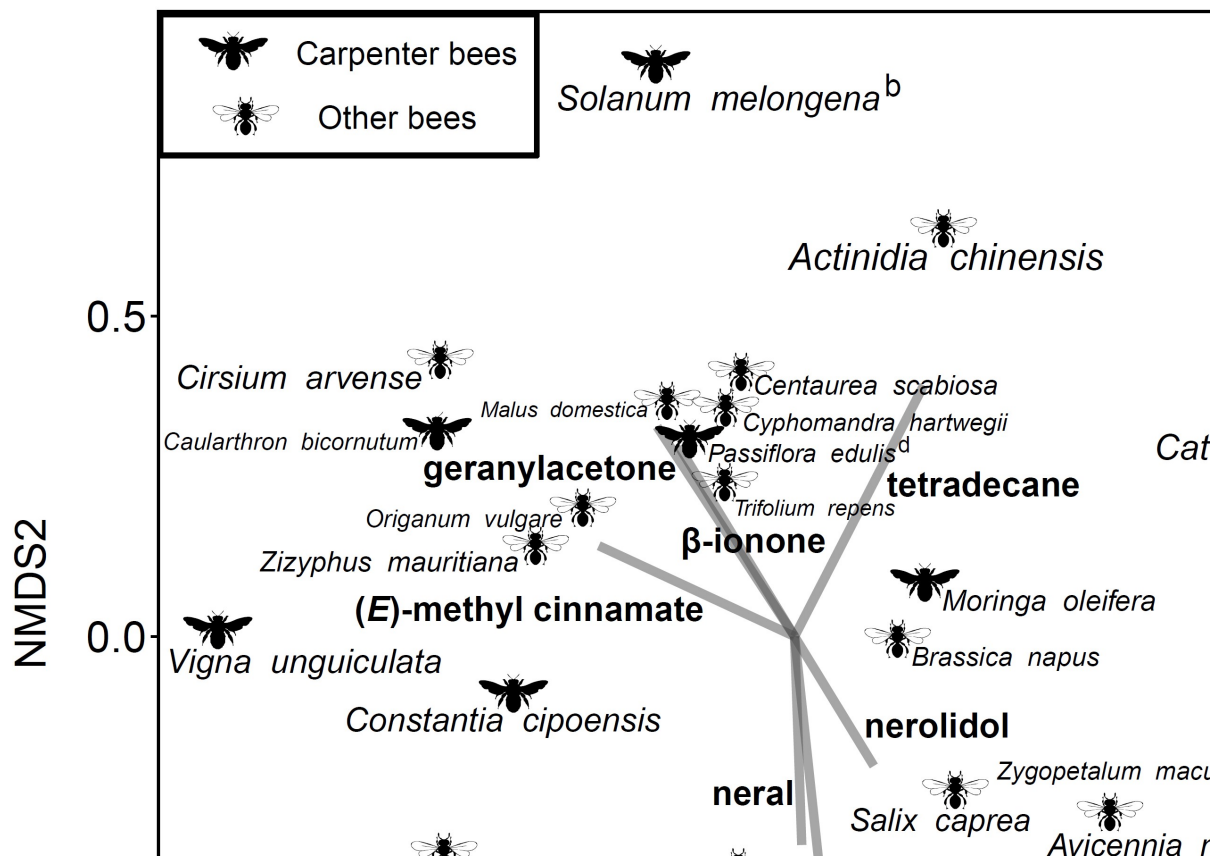
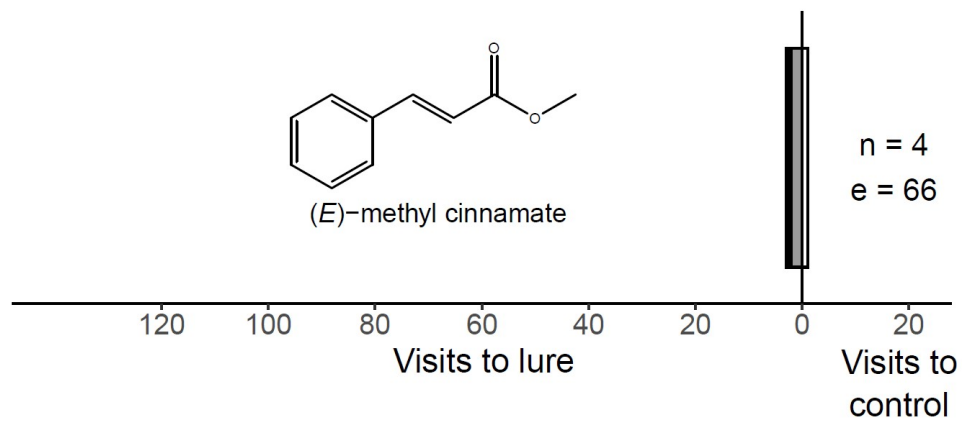
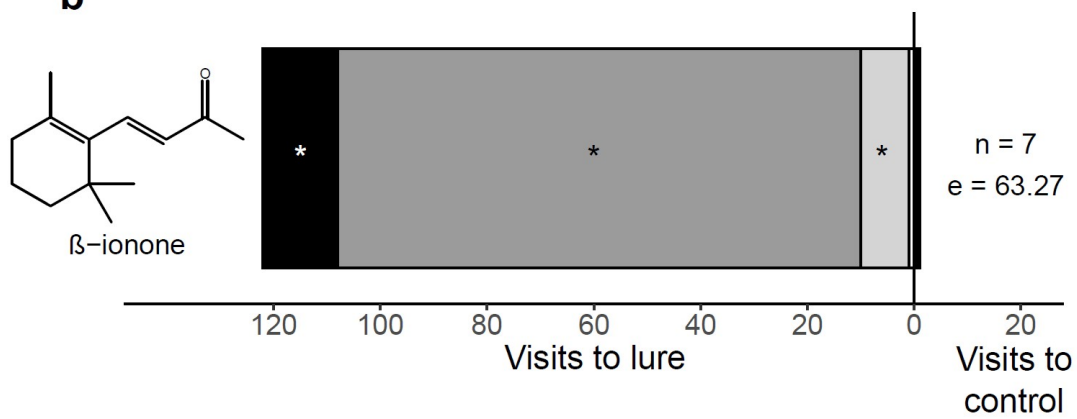
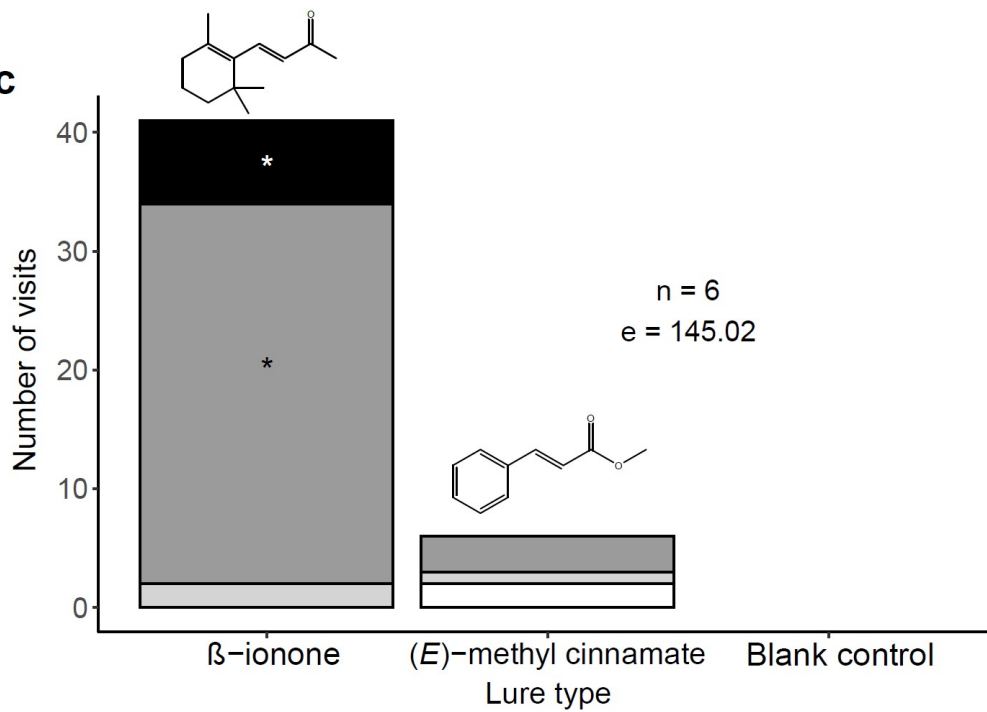


Fig. 1 Non-metrical Multidimensional Scaling (NMDS) ordinations of data on floral volatile organic compounds using Bray-Curtis distances with 28 of the 29 plant species studied (names in *italic*). The plot is built with the relative proportions of organic volatile compounds (in % of the total blend) and represents the relationships among species based on the dissimilarities of their floral volatiles. Vectors depict lines of maximum correlation of in the NMDS scores with relative abundances of the seven floral volatiles (names in **bold**) found to be indicative of pollination by carpenter bees in the Indicator Compound Analysis

a**b****c**

Carpenter bees
 Male euglossines
 Other bees
 Other insects

Fig. 2 Pollinator responses in two-choice (a and b) and multiple-choice (c) field assays with testing baits (filter paper impregnated with synthetic compound) and negative controls (only filter paper). **(a)** (*E*)-methyl cinnamate vs. control. **(b)** β -ionone vs. control. **(c)** β -ionone, (*E*)-methyl cinnamate, and control baits presented simultaneously. n = number of day replicates, with exposure of three to seven bait-control pairs or trios a day; e = sampling effort in baits.hours. Exact binomial (scent vs control in two-choice assays) and goodness-of-fit tests (equal probability of visit to all baits vs non-equal probability of visits in the multiple-choice assays) were performed only for the assays involving β -ionone: *, $P \leq 0.001$; pollinators with number of choices below five were not tested

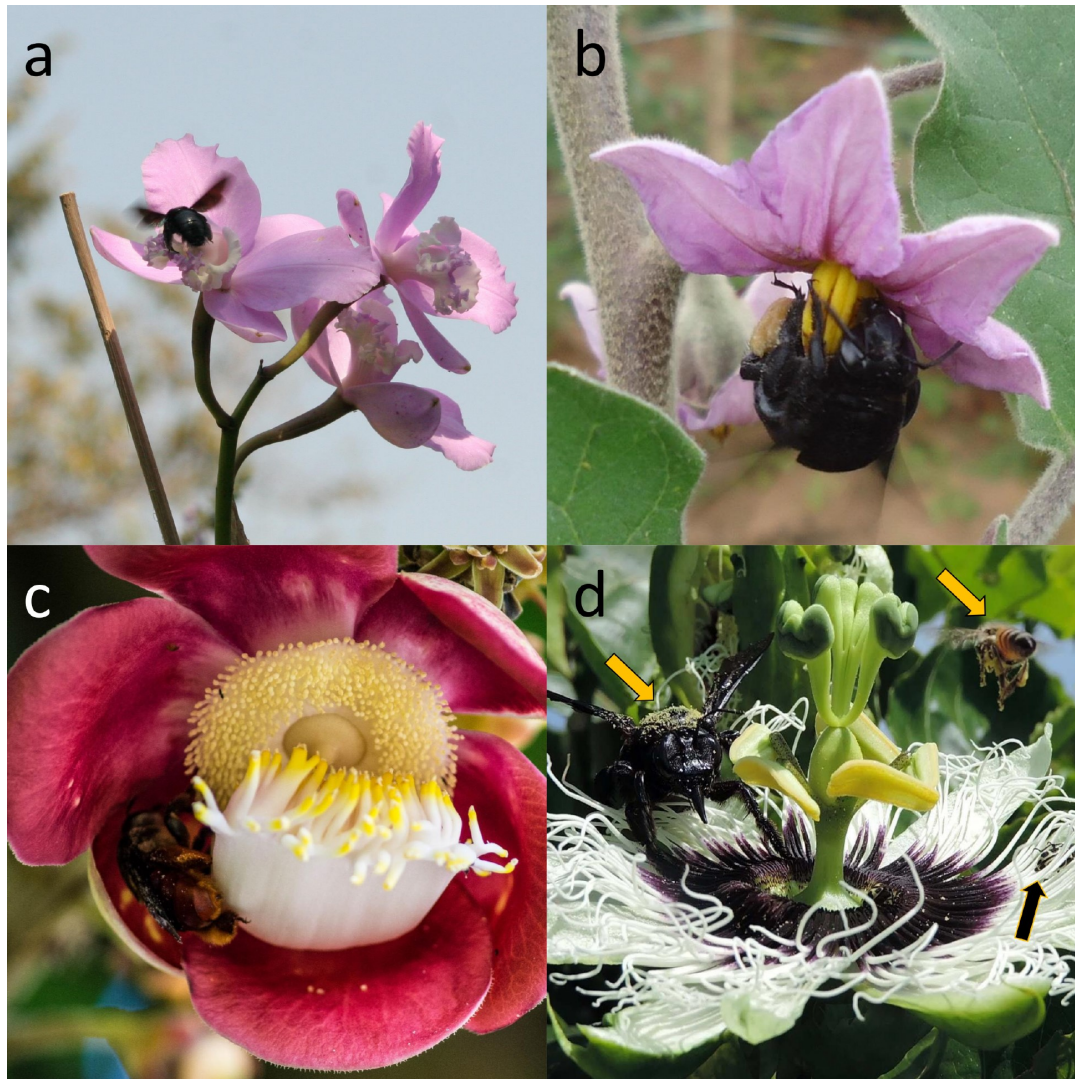


Fig. 3 Images of four plant species mainly pollinated by carpenter bees (*Xylocopa*) included in this study illustrating the diversity of traits other than floral volatiles in this guild. (a) *Cattleya loddigesii* and *Xylocopa* sp. (b) Eggplant, *Solanum melongena* and *Xylocopa* sp. (c) *Couroupita guianensis* and Centridini bee. (d) The passionflower *Passiflora edulis* simultaneously visited by a carpenter bee *Xylocopa* aff. *frontalis* (left arrow), a honeybee, *Apis mellifera* (top right arrow) and a Chrysomelidae beetle (bottom right arrow)

Supplementary Information

Online Resource 1 The 29 bee-pollinated plant species used in the work with their respective main pollinators and with an indication if the plant species is native from the study site

Online Resource 2 Percentages of the floral volatile organic compounds (FVs) in the 29 bee-pollinated plant species used in the work. For each plant species, there are the amounts of FVs (in %) categorized into main classes of compounds and then the amounts of each FV individually, with its respective number of registry on CAS (Chemical Abstracts Service of the Chemical American Society) and with the Retention Index (RI) associated to it in the articles used for data compilation or with the RI obtained in laboratory's identification in the case of *Cattleya loddigesii*