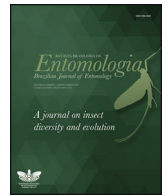




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Systematics, Morphology and Biogeography

Differences in volatile composition and sexual morphs in rambutan (*Nephelium lappaceum* L.) flowers and their effect in the *Apis mellifera* L. (Hymenoptera, Apidae) attraction



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ABSTRACT

We studied the volatile composition and sexual morphs of *Nephelium lappaceum* flowers from two orchards, and investigated the choice behavior of the honey bee, *Apis mellifera* toward the floral extracts from both locations. Our results showed significant differences in chemical composition and sexual morphs; only the hermaphrodite flowers from the Herradero orchard produced limonene and  $\alpha$ -pinene and had longer peduncle and sepal than flowers from the Metapa orchard; on the other hand, the hermaphrodite flowers from the Metapa orchard had longer gynoeceum. In the behavioral experiment the extracts from the Herradero orchard seemed to give *A. mellifera* foragers better cues for orientation to food sources, perhaps due to the presence of limonene and  $\alpha$ -pinene, which are absent in the samples from Metapa. Such differences in both orchards could affect pollinator attraction and ultimately seed set and productivity.

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Introduction

Pollination is an ecological interaction mediated by plants that produce flowers as a means to attract pollinators to reproductive organs. Actually plants exploit the learning capabilities of many insect pollinators by showing specific morphologies and producing particular floral volatiles that differentiate them from flowers of other species, triggering the so-called floral constancy behavior described in many insect species (Andrews et al., 2007; Whitney and Federle, 2012). For that reason, visually perceived characteristics are of utmost importance for pollination; for example, studies on the floral structure of *Geoffroea decorticans* (Gillies ex Hook. et. Arn) Burk, 1949 showed that variation in flower size and corolla length could affect pollinators' rate of visitation (Eynard and Galetto, 1999). Other studies have characterized floral volatile compounds in economically important crops, such as alfalfa (*Medicago sativa* L., 1753) (Pecetti et al., 2002; Pecetti and Tava, 2000), coffee (*Coffea arabica* L., 1753) (Vázquez et al., 2003), rambutan (*Nephelium lappaceum* L., 1767) (Mérida et al., 2003), onion (*Allium cepa* L., 1753) (Silva et al., 2003), pumpkin (*Cucurbita moschata* Duchesne ex Poiret, 1768) (Andrews et al., 2007) and canola

(*Brassica napus* L., 1753) (Wright et al., 2002). Floral volatiles have very important roles in attracting bees to reproductive organs, in which rewards like pollen, nectar or oils are present and strength floral constancy; therefore, any change in flower's scent, and also shape, could potentially affect the rate of visitation of pollinators, and hinder reproductive fitness (Byers et al., 2013; Jürgens et al., 2000; Nunes et al., 2016; Schäffler et al., 2015).

Most flowering crop plants depend on insect pollination for fruit and seed set; bees pollinate almost 90% of wild vegetation and 35% of crops (Klatt et al., 2013; Klein et al., 2007; Slaa et al., 2006). Therefore, any knowledge on the effect of floral cues on pollinators would allow us to increase fruit set and crop yield; unfortunately, few studies have aimed to explore such issue (Byers et al., 2013). For example, studies with strawberries and its main pollinator the solitary bee *Osmia bicornis* L., 1758 have shown that foraging behavior is deeply influenced by floral scent, though floral display also plays a role; more interestingly yet, differences in volatile emission rates between varieties of strawberries influence bee visitation rates under field conditions (Chagnon et al., 1993; Howell and Alarcon, 2007; Klatt et al., 2013). Studies performed with alfalfa (*Medicago sativa* L., 1753) floral volatiles and *Apis mellifera* L., 1758 demonstrated that linalool was the only compound attractive to honey bees, among five other antennally perceived compounds, at the optimized concentration (Henning et al., 1992). Thus, to gather a better understanding about the importance of

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floral volatiles on pollination of economically important crops it is necessary to explore the chemical-morphological and behavioral interactions of pollinators and their hosts.

In the tropics, rambutan, which is native to Southeast Asia, is a crop with major economic impact that is rapidly expanding into Southern Asia, Australia, the Caribbean, India, Sri Lanka, Florida, Hawaii and South and Central America (Rincón-Rabanales et al., 2015; Pohlan et al., 2007). Rambutan plants are trioecious with about 50% male and 50% hermaphrodite plants and in commercial orchards only hermaphrodite plants are grown, where most of them are functionally female. Recent studies in India found the stingless bee *Tetragonula iridipennis* Smith, 1854 and the Indian honeybee *A. cerana* Fabricius, 1793 to be the dominant visitors of *N. lappaceum*. In the Soconusco region, Chiapas, Mexico, rambutan was introduced during 1950–1970 as an economic alternative to coffee, mango and banana, but it has been successfully introduced into many more regions. Currently, rambutan is grown mainly in three municipalities: Cacahoatan, Metapa de Domínguez and Huehuetan (Rincón-Rabanales et al., 2015; Vanderlinden et al., 2004), in which *Scaptotrigona mexicana* Guérin-Meneville, 1845, a native bee, is considered as the main pollinator, otherwise beekeeping of *A. mellifera* also occurs. Nonetheless, there is a lack of information on floral volatiles, morphological traits and their interaction with honey bees. Therefore, our study aimed to explore and understand such interaction by: (a) determining the volatile composition of rambutan flowers from two different orchards, (b) measuring morphological traits in flowers from both locations and (c) evaluating honeybee attraction to rambutan floral volatiles.

## Materials and methods

### Study site and volatile collection

This study was carried out in two orchards located 1.5 km from each other in Metapa de Domínguez in the Soconusco region: Metapa (14°50' N, 92°11' W, 100 masl) and Herradero orchards (14°49' N, 92°11' W, 100 masl). In both orchards the origin of plants can be traced back to one single donor plant; nonetheless despite the clonal origin, evident differences in morphological traits are present, such as flower shape and size. From February to March 2015 in each orchard we collected one panicle with hermaphrodite flowers per tree ( $N=6$ ); we wanted to investigate any potential difference between male and hermaphrodite flowers, thus in Herradero orchard we collected two panicles with male flowers per tree ( $N=2$ ), unfortunately we did not find male flowers in Metapa orchard. All sampled panicles were newly opened and randomly chosen by a blind (collector did not know the final fate of the flowers, either for morphometric or volatile analysis) procedure from the middle part of the canopy. All panicles were carefully removed, separately transferred in labeled polyethylene bags and transported in a cooler at 8 °C to the laboratory of Chemical Ecology in *El Colegio de la Frontera Sur* (ECOSUR). Twenty to 30 min after sampling, floral volatiles were collected with a solid phase microextraction (SPME) technique; the SPME syringe consisted in a polydimethylsiloxane-divinylbenzene fiber of 65  $\mu\text{m}$  thick, the fiber was inserted for 30 min in a glass vial (7 mL) where flowers could not touch it. Next, the SPME fiber were immediately analyzed in a gas chromatograph coupled with a mass spectrometer Varian Saturn 2200 (Sandoval et al., 2007) with a DB5-MS nonpolar capillary column containing 95% of dimethylpolysiloxane and 5% of diphenyl siloxane, with a length of 30 m long by 0.25 mm inside diameter and 25  $\mu\text{m}$  thick film, using helium as a carrier gas. Samples were analyzed by an initial program of 50 °C for 2 min with an increase of 15 °C/min to a temperature of 280 °C for 10 min. The injector temperature was 250 °C.

Volatiles were identified by comparing Kovats index, mass spectra and retention times of synthetic standards in conjunction with the National Institute of Standards and Technology library version 2.5 (NIST). All chemicals were acquired at Sigma-Aldrich Chemical, Parque Industrial Toluca 2000, street 6 north N° 107, P.C. 50200 Toluca, Mexico.

### Morphological analysis

For this analysis we randomly chose one newly opened panicle per each of three plants in each orchard and were transported to the laboratory as described lines above; next, we randomly chose 50 flowers per site and removed them from the panicle. Immediately after separation from the panicle, sepal, gynoecium, peduncle length, calyx diameter and calyx width were measured using a calibrated scale and a digital camera (Carl Zeiss AXIO CAM MRC, E206588, Zen software 2011, LLC, United States) mounted on a stereomicroscope (Carl Zeiss model STEMI 2000c, LLC, United States).

### Behavioral test

Foragers from two healthy queenright *A. mellifera* colonies were evaluated from November 2015 to January 2016 at 9–14 h in the campus of *El Colegio de la Frontera Sur*. The flower extracts used in this experiment were obtained by taking 800 hermaphrodite flowers of each orchard and separately washed for 30 min in 250 mL of hexane and taking it to a final volume of 100  $\mu\text{L}$  using nitrogen; these extracts showed a chemical profile similar to the extracts obtained by SPME. The experiment consisted of two sequential phases. *Training phase*: Five to ten foragers were trained to collect 2 M sucrose solution from a feeder located at 10 m from the colony under study (Sánchez et al., 2008). The feeder consisted of a 1.5 cm diameter cotton ball soaked in the sucrose solution and placed on a plastic Petri dish, with a filter paper inside, which was used to release the floral volatiles. Trained foragers were distinctively paint-marked on their thorax with water-based paint to distinguish them from newcomers. Since there were feral colonies in the vicinity of our setup, we paint-marked the thorax of the foragers of our colony with a device that allowed us to distinguish them from those foragers coming from other colonies and from the trained ones (Mikery-Pacheco et al., 2013). *Test phase*: Once at the experimental distance, the training feeder was removed and two clean feeders separated from each other by 70 cm were showed to the foragers. One feeder contained a piece of filter paper with 100  $\mu\text{L}$  of the extract of one of the orchards and the other one with a piece of filter paper with 100  $\mu\text{L}$  of hexane. For 20 min any unmarked or device-marked forager that started collecting the sucrose solution was trapped using an entomological aspirator until the end of the experiments to avoid pseudoreplication, and its choice was registered; feeders exchanged places every 5 min to avoid site-learning. Besides, we registered the choices of the trained foragers, but they were not trapped to keep bringing newcomers to our setup. Overall, we carried out three replicates per colony per each extract orchard; each replicate was performed on a daily basis.

### Data analysis

We built classification trees by analyzing the data from volatile composition, flower sex and study sites using the randomForest method from the randomForest package (Liaw and Wiener, 2002) implemented in R software; this analysis finds associations between individuals through regression using a permutation approach, which allows the analysis of small sample sets.

Morphometric data were analyzed by a Kruskal–Wallis test to compare sepal, petal length and gynoecium height between the

**Table 1**  
Compounds identified in rambutan hermaphrodites and male flowers from Herradero and Metapa orchards.

No.	RT	RI	Compounds	Hermaphrodites		Males
				Herradero	Metapa	Herradero
1	5.787	895.524	$\alpha$ -pinene	0.98 $\pm$ 0.71		
2	6.080	1012.609	D-Limonene	4.93 $\pm$ 4		
3	6.335	1037.342	Trans- $\beta$ -ocimene	12.83 $\pm$ 8.03	17.87 $\pm$ 6.88	22.15 $\pm$ 7.15
4	7.924	1189.809	Cis-3-hexenyl butyrate	1.46 $\pm$ 0.83	3.39 $\pm$ 1.20	15.69 $\pm$ 4.05
5	8.152	1212.525	Decanal	5.90 $\pm$ 2.36	2.38 $\pm$ 0.94	
6	10.426	1462.573	Trans-geranyl acetone	7.97 $\pm$ 2.64	1.49 $\pm$ 0.51	
7	10.855	1513.982	$\alpha$ -farnesene	57.33 $\pm$ 6.44	50.02 $\pm$ 5.39	62.15 $\pm$ 3.10

RT, retention time; RI, retention index; Traces amount (Mean  $\pm$  Standard Error).

two orchards, respectively ( $<0.05$ ) with SAS software v9.1.3 (SAS Institute, Carry, NC, USA). All statistical tests were performed in Statistica version 7.1 (StatSoft, 2005). Behavioral data were analyzed with an exact Wilcoxon signed rank test for paired data using package exactRankTests v0.8 (Hothorn and Hornik, 2015) with software R v3.02 (RCoreTeam, 2015).

## Results

### Chemical analysis

Overall a total of seven volatile compounds were identified. The compounds identified in male flowers were Trans- $\beta$ -ocimene, Cis-3-hexenyl butyrate and  $\alpha$ -farnesene, which were present in El Herradero orchards; we did not find male flowers in the Metapa orchard. Hermaphrodite flowers from Herradero had D-limonene and  $\alpha$ -pinene, which were absent in Metapa. The major compound was  $\alpha$ -farnesene, which was present in both sexes and orchards (Table 1). Fig. 1 shows the grouping of the panicles according to the compounds we detected.

### Morphological analysis

Floral characteristics showed differences between both orchards. All three peduncle, gynoecium and sepal length were significantly different, whereas calyx diameter and calyx width were

not. Herradero orchard flowers were longer in peduncle ( $p < 0.001$ ,  $F_{48}$ ) and sepal length ( $p < 0.033$ ,  $F_{48}$ ), nevertheless in Metapa orchard, flowers were longer in gynoecium length ( $p < 0.001$ ,  $F_{48}$ ). Table 2 shows descriptive statistics on morphometric data.

### Behavioral tests

We found that significantly more newcomers chose the Herradero extract ( $36 \pm 2.3$  foragers) than the control-hexane ( $24.16 \pm 2.3$  foragers) ( $V = 0$ ,  $p = 0.031$ ); on the other hand, the Metapa orchard extract and the control-hexane did not show any significant difference ( $12.71 \pm 2.3$  foragers extract;  $15.14 \pm 2.3$  foragers control;  $V = 12.5$ ,  $p = 0.718$ ). Fig. 2 shows the preference of the bees in the behavioral tests. In the case of trained, experienced foragers no difference was found in choice behavior for both orchards.

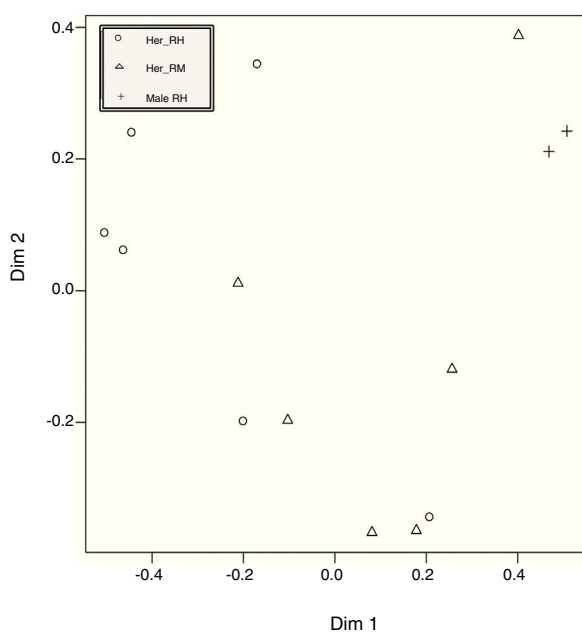
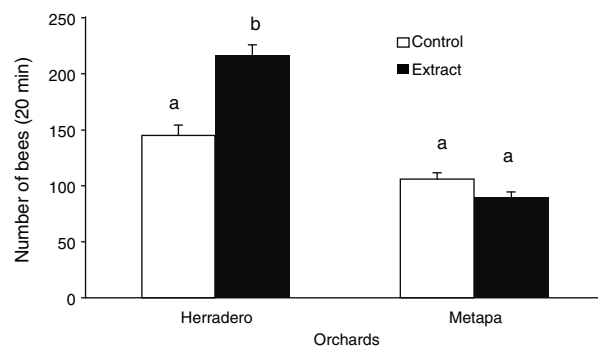
## Discussion

Most of the floral scent related studies have focused on inter-specific variation of these signals so far, but very little is known about intraspecific variation related to geographic distributions. Our study revealed that rambutan flowers from Herradero and

**Table 2**  
Morphological traits of rambutan hermaphrodites flowers from Herradero and Metapa orchard (Mean  $\pm$  Standard Error).

Morphological traits	Orchard	
	Herradero (mm)	Metapa (mm)
Sepal length	1.59 $\pm$ 0.03 <sup>a</sup>	1.50 $\pm$ 0.04 <sup>b</sup>
Gynoecium length	2.27 $\pm$ 0.04 <sup>b</sup>	2.54 $\pm$ 0.09 <sup>a</sup>
Peduncle length	1.32 $\pm$ 0.04 <sup>a</sup>	1.04 $\pm$ 0.03 <sup>b</sup>
Calyx diameter	2.35 $\pm$ 0.04 <sup>a</sup>	2.30 $\pm$ 0.05 <sup>a</sup>
Calyx width	2.54 $\pm$ 0.03 <sup>a</sup>	2.58 $\pm$ 0.06 <sup>a</sup>

Different letters in the same line mean statistical differences ( $p < 0.05$ ).

**Fig. 1.** Relation between compounds in rambutan hermaphrodite flowers from Herradero (Her\_RH) and Metapa orchard (Her\_RM), and male flowers (Male\_RH).**Fig. 2.** Behavioral tests with orchards extracts (Herradero and Metapa; dotted bar) against control (white bar). The Y-axis represents the total number of bees over 20 min of observations (six replicates per orchard). Different letters above each pair of columns mean a significant difference ( $p < 0.05$ ).

Metapa differed both qualitatively and quantitatively in their scent profiles. Such intraspecific flower scent variation is observed in a limited number of examples, e.g. in specimens of *Silene latifolia* Poirlet, 1789 where quantitative and qualitative variability was observed in European and North American populations (Dötterl et al., 2006). This was also registered in the highly specific plant-insect pollination interaction, fig – fig wasp mutualism, where significant differences were found between East Asian and Indian populations (Soler et al., 2011). In regard to qualitative differences, our results showed that two of the volatiles produced by Herradero flowers; limonene and  $\alpha$ -pinene, were absent in Metapa flowers. This intraspecific variation was also reported in three *Salix* species, which could be related to sex differences (Füssel et al., 2007). In this sense, our data showed that rambutan male flowers differed with hermaphrodite ones, by registering only three compounds: ocimene, butanoic acid and  $\alpha$ -farnesene. Most of the compounds found in hermaphrodite and male rambutan flowers have also been reported as part of the floral odor bouquet in other angiosperms that are important in a variety of plant-pollinator interactions, e.g. strawberry flowers (*Fragaria*  $\times$  *ananassa* Duchesne, 1788) emit (E,E)- $\alpha$ -farnesene, limonene, p-anisaldehyde, (Z)-3-hexenyl acetate, methyl salicylate, benzaldehyde, which ones are known to be attractants to honeybees (Klatt et al., 2013). In *Mimulus lewisii* Pursch, 1774 flowers D-limonene,  $\beta$ -myrcene and  $\beta$ -ocimene, are the most important volatiles driving bumble bee flower visitation rate (Byers et al., 2013). In lemon flowers, the major compound was limonene (Grajales-Conesa et al., 2012). *Fragaria virginiana* Miller, 1768 and *Euphorbia cyparissias* L., 1753 emit floral scents made up of similarly widespread compounds, including linalool,  $\beta$ -caryophyllene,  $\alpha$ -terpineol,  $\alpha$ -pinene and (E)- $\beta$ -ocimene, which are crucial at attracting potential pollinators during daytime, mostly ants (Vega et al., 2014). Hence, scent is one of the floral traits particularly important in driving pollinator behavior and mediating reproduction in flowering plants (Byers et al., 2013).

The intraspecific variation of floral scent compounds could result of relaxed selective pressure, genetic drift, introgression of scent traits through hybridization, pleiotropic effects of plant defense on scent biosynthesis, or phenotypic plasticity resulting from edaphic or climatic differences (Raguso, 2008). Floral scent often operate synergistically with floral visual display, and morphological traits contribute to effective resource partitioning (Byers et al., 2013); for example, differences in proboscis length in bumble bees allow foragers with longer proboscis to collect from flowers with longer corollas (Johnson, 1986), and differences in body coloration in the *Melipona* genus allow some species to forage in sunnier, warmer conditions (Biesmeijer et al., 1999). In our study, we found that hermaphrodite flowers from the Herradero orchard had longer peduncle and sepal than flowers from Metapa orchard, which were longer in gynoeceum. In natural populations of *Echinopsis ancistrophora* Spegazzini, 1905 flower length was associated with floral traits of visual display, reward presentation and pollinator fit (Schlumpberger et al., 2009).

Pollinators such as honey bees are capable of learning the scent of a plant species and forage almost exclusively on it provided it is delivering nectar and pollen (Wright et al., 2005). Our results showed that Herradero floral extract was more attractive than control; however, Metapa floral extract was not. Fidelity of honey bee foragers to Metapa orchard thus could be better explained by site constancy (Grüter et al., 2011) than floral constancy itself, as a result that the bees did not discriminate between extract and hexane and they just learned the resource location. Honey bees can discriminate among the scents of different cultivars and recognize subtle differences in scent, e.g. with three varieties of snapdragon (*Antirrhinum majus* L., 1753) bees differentiated the odor of one variety from the odor of other varieties, which suggests that scent intensity,

the types of compounds present and the number of common odors that a bee has previously experienced affect the ability of honeybees to recognize and differentiate among cultivars and varieties (Wright et al., 2005; Wright et al., 2002). This difference might be related to presence and concentration of odor signals, which vary from flower to flower, therefore slight changes in volatile composition could cause the rejection of certain flowers once is perceived by the pollinator. During the lifespan of a flower, changes in scent composition may be caused by both environmental factors such as light intensity and temperature, or biological traits such as genetic differentiation or circadian rhythms of scent emission, floral age, and pollination status which may influence pollinator discrimination (Wright et al., 2005). Our study provides a first step to understand how floral fragrances, morphological traits and sexuality mediate bee attraction to rambutan flowers. Nonetheless, there is a need to evaluate identified compounds or the mix of them inside the orchards with rambutan pollinators, to prove which ones are more attractive for them.

### Conflicts of interest

The authors declare no conflicts of interest.

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