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Floral scent variation in two *Antirrhinum majus* subspecies influences the choice of naïve bumblebees

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Abstract Two wild subspecies of snapdragon, *Antirrhinum majus*, subspecies *pseudomajus* and *striatum*, differ in floral color and can be visually discriminated by insect visitors. The extent to which olfactory cues derived from floral scents contribute to discrimination between snapdragon subspecies is however unknown. We tested whether these two subspecies differ in floral scent and whether these olfactory differences are used by bumblebees (*Bombus terrestris*) to discriminate between them. We grew individuals of both subspecies, collected from a total of seven wild populations, under controlled conditions. We quantified the volatile organic compounds (VOCs) emitted by the flowers using gas-chromatography/mass-spectrometry/flame-ionization-detection. We studied antennal detection of VOCs by bumblebees, by means of electroantennogram study (EAG). We also performed behavioral experiments in a Y-maze to determine the innate response of bumblebees to the main floral VOCs emitted by our snapdragon subspecies. The floral scent of *Antirrhinum majus pseudomajus* contained three volatile benzenoids absent in the floral scent of *Antirrhinum majus striatum*. One of them, acetophenone, contributed over 69% of the absolute emissions of *A. majus pseudomajus*. These benzenoids elicited a significantly higher EAG response compared with other VOCs. In the Y-maze, bumblebees were significantly less attracted by acetophenone, suggesting an aversive effect of this VOC. Our findings indicate that bumblebees are able to discriminate between the two *Antirrhinum majus* subspecies. Differences in flower scent between these subspecies and olfactory bumblebee preferences are discussed in the light of biochemical constraints on VOCs synthesis and of the role of flower scent in the evolutionary ecology of *A. majus*.

Keywords Snapdragon · *Antirrhinum majus* · Bumblebee · *Bombus terrestris* · Flower scent · Olfactory preference

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Introduction

Plants have evolved a fascinating array of cues to attract animal pollinators and this has been a fertile ground for

research in evolutionary ecology (Ricklefs and Renner 1994; Mitchell et al. 2009). The mechanisms of flower detection by pollinators are complex, and understanding them also is an active field of research (Giurfa and Vorobyev 1997; Chittka and Thomson 2001; Harder and Barrett 2006). Insect-pollinated flowers offer visual cues through flower size, shape, and color, which are used by the pollinators to find and discriminate their resources (Giurfa and Menzel 1997). The volatile organic compounds (VOCs) of the flower scent also play an important role in plant–pollinator interactions (Stebbins 1970; Heinrich and Raven 1972; Raguso 2008), and new techniques in organic chemistry have shed a new light on the role of these VOCs (Tholl et al. 2006). For instance, pollinators may be innately attracted by floral VOCs (Omura and Honda 2005; Riffell et al. 2008) and also learn to associate a scent with a food reward (Komischke et al. 2002; Deisig et al. 2002; see Giurfa 2007 for a review).

In the mountain range of the Eastern Pyrenees, two wild subspecies of *Antirrhinum majus* (Scrophulariaceae) are encountered on roadsides and open habitats. The subspecies display characteristic flower colors: the flowers of *Antirrhinum majus pseudomajus* are magenta, whereas *Antirrhinum majus striatum* has yellow flowers (Fig. 1). The flowers are self-incompatible (an estimated 4% of selfing), and they are thought to be pollinated by relatively large Apidae, as these have strong enough bodies to open the closed corolla (Andalo et al. 2010). A nectar reward is offered to flower visitors. In a preliminary census of flower visitors in our study site, we inventoried over ten species of insect visitors in the genera *Bombus* and *Xylocopa* with *Bombus terrestris* being the most frequent species (Suchet et al. unpublished data). These insects were observed as flower visitors in both snapdragon subspecies. The subspecies are usually allopatric or parapatric, but they are occasionally found in contact, and there pollinators cross-fertilize the flowers, generating hybrids that display a wide array of coloration patterns (Whibley et al. 2006). Whibley et al. (2006) found that, in the hybrid zone, flower color was under strong stabilizing selection, and they suggested that pollinators may counter-

select the hybrids. In support of a pre-zygotic barrier to gene flow, bumblebees were shown to discriminate between the two colors of subspecies (Tastard et al. 2008). However, in experiments with artificial flowers with the colors of the two parent lines, the constancy of the visitor was only weakly associated with flower color (E. Tastard, C Andalo, C Thébaud et al., unpublished results). Thus, no direct link between selection on the floral phenotypes and the color of these phenotypes was demonstrated.

In addition to color differences, it is also possible that VOCs differ between the flowers of the subspecies of *A. majus*, and if so, that pollinators use flower scent as a detection and recognition cue. In the present work, we thus study the role of floral VOCs in the detection of *A. majus* by bumblebees. The domesticated relatives of the wild *A. majus* (horticultural snapdragon) have long served as a model species to discover the chemical pathways of floral volatiles (Dudareva et al. 2000, 2003, 2006). By artificially manipulating floral scent of horticultural snapdragons in free-flying bumblebee studies, Odell et al. (1999) showed that it is the color that influences the bumblebee behavior in the tested floral color–scent combinations. However, Wright et al. (2005) showed that honeybees could discriminate slight changes in the composition of a mixture of VOCs using snapdragon cultivars. Focusing on horticultural snapdragons, these studies are therefore conflicting. However, the floral scent of horticultural snapdragon may differ from that of their wild relatives. We here study the composition of the floral scent of the two wild *A. majus* subspecies. In addition, we assess how bumblebees may show innate preferences for one or the other scent.

In the present work, we combine VOC identification, biologically active VOCs determination, and behavioral tests with the interacting insect because they are important steps to understand chemical mediation in plant–insect interactions (Schatz et al. 2009). Our questions are the following: (1) Do the two subspecies *A. majus pseudomajus* and *A. majus striatum* produce distinct floral blends of VOCs under identical greenhouse conditions? (2) Can naïve bumblebees perceive most of the VOCs of the two

Fig. 1 Floral phenotypes of the two subspecies of the wild *A. majus* species, *A. majus striatum* (*left*), and *A. majus pseudomajus* (*right*)

A. majus subspecies flower scent? (3) When exposed to simulated floral scent of the two subspecies, do naïve bumblebees show a preference toward one scent?

Materials and methods

Plant material growth and collection

A. majus is a widespread semi-perennial plant (Scrophulariaceae), with annual axes of racemous inflorescences and zygomorphic flowers, growing at an elevation of 0–1,600 m asl in the Eastern Pyrenees mountains. The distribution of the two subspecies is non-overlapping, except for a few hybrid zones (see map in Whibley et al. 2006). Flowers are closed and self-incompatible and are visited mostly by Apidae (Andalo et al. 2010). Even though the pollinating efficiency of flower visitors has not been studied in detail, *Bombus* species (henceforth bumblebees) are pollen vectors for *A. majus* as we did observe fresh pollen deposited on their thorax and legs after flower visitation (C Suchet, personal observations).

We grew ten seeds from each of four wild populations of *A. majus pseudomajus* near the villages of Lagrasse, La Preste, Le Martinet in France, and near Pardines in Spain (for a total of N_p=40 adults), and from each of three wild populations of *A. majus striatum* near the villages of Lles, Collada de Toses in Spain, and Camurac in France (for a total of N_s=30 adults). The populations were selected so as to maximize their geographical range and elevation with respect to the other subspecies. Initially, a fourth population of *A. majus striatum* was included in our experimental design, but germination showed a low rate of success, and it was subsequently discarded. Seeds were collected between 2000 and 2006 at the end of the growing season from mature fruits. Seeds from ten fruits from ten individual plants for each of the seven populations were grown in greenhouse conditions between November 2008 and May 2009 (16 h/day of light, at 25°C average temperature, in individual pots with universal compost and with no addition of nutrients). Ten adult plants were selected by population at the flowering time for their similar phenotypes and their diversified genetic sources. We minimized the number of sampled plants grown from the seeds of the same fruit. Overall, five to seven maternal lineages are represented among the ten selected plants by population.

Determination of floral scents

Sampling of floral scent The VOCs emitted by the flowers were sampled between February and May 2009 in the greenhouse. Preliminary analyses showed that diurnal variations in emission were similar with those described by Dudareva et al. (2000, 2003). Therefore, sampling was conducted during the peak of emission intensity, between 11:00 AM and 4:00 PM, and on whole inflorescences. To minimize biases due to flower developmental stages (Dudareva et al. 2000, 2003; Goodwin et al. 2003), VOCs were sampled when the inflorescence had at least four open flowers with dehiscent anthers.

To sample floral emissions, a dynamic headspace method was used (Tholl et al. 2006). We enclosed each inflorescence in vivo into a 2-L glass chamber, and the VOCs were adsorbed on a TenaxTA 60/80 (100 mg) trap connected to a battery-operated vacuum pump operated at 200 mL min^{-1}. This design optimizes the signal/threshold ratio without exceeding the breakthrough volumes of each VOC (Kesselmeier et al. 1996; Simon et al. 2005a, b). The flow rate that purges air from the headspace was maintained at 600 mL min^{-1}. Sampling duration was fixed at 10 min. To control for possible environmental contamination, ambient air was also trapped during each sampling session. Sample tubes were stored in the dark at 0–4°C before analysis.

Floral VOC emissions depend on light intensity and temperature (Guenther et al. 1995; Dudareva et al. 2000, 2003, 2006). Hence, we measured both variables from the headspace of the inflorescence for each sampling session. Temperature was measured with an EL-WIN-USB datalogger (Lascar Electronics LTD., UK). Photon flux was measured with a LI250A light meter connected to a LI190SA Quantum Sensor (LI-COR Biosciences, Lincoln, USA). The intrafloral temperature may differ among flowers of different color in *A. majus* (Comba et al. 2000), but we did not measure this parameter because of technical difficulties. To normalize the emissions among plants, we cut the inflorescences after VOC sampling, and we measured their oven-dry weight (inflorescences were dried at 100°C for 48 h).

Analyses of volatile compounds The VOC samples were thermodesorbed using a Turbomatrix TD desorber (Perkin-Elmer, USA) and were analyzed using a gas chromatograph coupled with a mass spectrometer and a flame-ionization detector (FID; Clarus 500, Perkin-Elmer, USA). The separation of VOCs was performed using a DB-5 non-polar capillary column (30 m×0.25 mm ID×0.25-μm film thickness). Oven temperature was held at 35°C for 5 min, heated to 160°C at 5° min^{-1}, and then up to 220°C at 15 min^{-1}. The carrier gas was helium. Mass spectra were recorded in the electron impact mode at an ionization voltage of 70 eV and scanned from m/z=33 to 450.

The identification of VOCs was based on their Kovats index relative to C_5—C_{18} *n*-alkanes and mass spectra which were matched with those from the NIST library (2005) and those reported in literature (Adams 2001).

The quantification of the compounds was made based on their FID peak area. Ocimene (TCI Chemical®, Stockholm,

Sweden, 90.0%) and nonanal (Extrasynthese SAS®, Genay, France, pure) were used as external standards. The calibration was carried out in laboratory conditions by injecting a liquid volume of standard solutions directly into the sample tube. A linearity range from 2×10^{-5} to 9.2×10^{-4} μg was observed for the two external standards ($R^2 = 0.99$ for both compounds). The theoretical response factor of the studied compounds was computed using the theory of the effective carbon number (Jorgensen et al. 1990). To quantify the VOCs that were not calibrated individually, we applied corrections to the mean response factors (Komenda et al. 2001).

The emission rate of each VOC was obtained from the difference between the quantity of compounds recorded inside and outside the glass chamber. The emission rate E [micrograms per gram (dry flowers weight)$^{-1}$ per hour] was computed using the following equation:

$$E = \left(\frac{m_2}{q_2} - \frac{m_1}{q_1} \right) \frac{Q}{Mt}$$

where m_2 and m_1 are the mass of the compound in the outlet and inlet flow rates (in micrograms), and q_2 and q_1 are the outlet and inlet flow rates (in milliliters per minute). Q is the flow rate of the enclosure purge air (in milliliters per minute), M is the dry weight of the enclosed flowers (in grams), and t the sampling time (in hours; Sabillon and Cremades 2001). This quantification method makes it possible to compare among the plants in our study because it normalizes the amount of VOCs to the dry flower weight, regardless of the difference in flower number per inflorescence. A uniform sampling and analytical uncertainty of ca. 30% is associated with the chamber design (Moukhtar et al. 2005).

Bumblebees' olfactory tests

B. terrestris *B. terrestris* was found to be the most frequent flower visitor of *A. majus* in the wild (Appendix 1). Commercial colonies of this species are available so that physiological and behavioral measures could be performed in the laboratory. Naïve individuals were chosen to perform both electroantenograms and behavioral tests with chosen chemical compounds (one colony purchased from Koppert®, Berkel en Rodenrijs, The Netherlands). We used only workers in our tests.

Electroantennography We determined whether VOCs present in floral emissions could be detected by the olfactory receptors located on the bumblebees' antennae. To this end, we performed electroantennogram recordings. Electroantenno-grams measure the summed response of olfactory receptors on an insect's antenna to a given olfactory stimulus (Roelofs 1984). Hence, they indicate whether an insect has the ability to detect such a stimulus.

A single bumblebee antenna was cut and fitted both ways into two glass pipettes filled with KCl solution and connected to the silver electrodes of an electroantennogram detector (SYNTECH®, Kirchzarten, Germany). The antenna was then stimulated with a VOC and responses (in volt-meter) were measured by means of a volt-meter via the electrodes as described and illustrated in Thiéry and Marion-Poll (1998). Ten synthetic VOCs detected in the floral scents of *A. majus* were tested: three benzenoids (benzaldehyde, methyl benzoate, and acetophenone, Sigma-Aldrich®, Bellefonte, USA,), the VOCs contributing the most to the differences between the two snapdragon subspecies (see below). We also included seven VOCs that were found to be either the most frequent and/or the most abundant in the two *A. majus* subspecies: cis-ocimene, limonene, nonanal, 2-butanone, myrcene, penta-nal, and hexanal (Sigma-Aldrich®, Bellefonte, USA; TCI Chemical®, Stockholm, Sweden; and Extrasynthese®, Genay, France). We decided to use synthetic VOCs rather than scents from real flowers so as to minimize the variability among the tests.

For each stimulus, 1 μl of pure solution was deposited on a strip of filter paper and left for evaporation during 30 min in ambient conditions in a separate laboratory room. We initially used pure solutions because we aimed to compare the amplitudes of the antennal activities among the perceived VOCs, but we also carried out controls with compounds diluted at 0.1%, 1%, 10%, and 50%. The antenna was stimulated with pulses of 0.5 s each, using a purified and moistened airflow of 11.3 mL s^{-1} across a Pasteur pipette containing the filter paper.

Each of the ten VOCs was delivered once; VOCs were presented in the same order so that the ten tested antennae were submitted to a similar stimulus sequence, thus allowing comparisons between antennal signals. Stimuli were separated by a 40-s interval, to avoid saturation of the olfactory receptors. A control was performed at the beginning of each experimental series by measuring the antenna response to clean air. To check the sensitivity of antennal responses throughout the sequence of stimuli, the VOCs for which the response was the most intense were assayed again at the end of the sequence. For each bumblebee antenna, the response amplitude was normalized to the maximum response recorded.

Spontaneous olfactory preferences in a behavioral assay To determine whether bumblebees exhibit spontaneous preferences when confronted with the VOCs of the two *A. majus* subspecies, we tested their choice in a Y-maze presenting a dual olfactory stimulation (Dupuy et al. 2006). In these experiments, we used naïve bumblebees from a colony raised in the laboratory, so their choices reflect innate preferences for olfactory stimuli.

The maze presented a main channel and two bifurcating arms. The main channel was 7 cm long and the two arms

were 14 cm long; all parts were 4 cm in height. Air flow moistened and neutralized by means of active carbon was delivered at the two extremities of the arms (flow rate, 150 mL min^{-1}). To favor a constant directional air flow through the maze, air was also pumped out at the entrance of the maze (flow rate, 200 mL min^{-1}).

To familiarize the bumblebees with the experimental set-up, we pre-trained them to the maze before testing them with VOCs. Bumblebees stayed 6 h in the dark, without food in ventilated individual plastic tubes. Each individual was then released in the entrance arm of the maze. During the pre-training stage, 20 µl of 50% sugar solution was offered at the intersection point of the maze. The bumblebee could freely move within the maze, and it easily found the drop of sucrose solution and consumed it. After this training, the bumblebee was replaced into its plastic tube and taken out from the maze. Between tests, the Y-maze was carefully cleaned using an ethanol solution. After a second pre-training visit, the experienced bumblebee was motivated to search for sucrose solution within the maze, and the olfactory tests could then begin. Both pre-training and test sessions were carried out in the dark, to favor the bumblebees' use of olfactory cues. To be able to observe the bumblebee behavior in real time, these tests were performed under red light. Because of the darkness, bumblebees were walking in the Y-maze.

In the olfactory tests, two olfactory stimuli were delivered, each coming from one of the two arms of the Y-maze. The VOCs were deposited on a 1-cm^2 piece of filter paper, and they were allowed to evaporate for 1 h in a separate laboratory room. The filter paper was then inserted in a 10-µL micropipette tip. In each arm of the Y-maze, the micropipette tip was inserted in a hole in the floor created for this purpose (Dupuy et al. 2006). Air filtered by active charcoal and moistened was pulsed at 150 mL min^{-1} from the dead end of each arm through Teflon tubes, allowing the olfactory stimuli to flow towards the decision area, defined as the area where the bumblebee had to make a choice. The bumblebee did not find any reward at the intersection of the arms during the test.

The olfactory stimuli presented to the bumblebees were either single synthetic VOC or mixtures of pure synthetic VOCs. Synthetic mixtures were used because VOC concentration could then be easily manipulated, so as to identify which VOC mostly influences bumblebee behavior. We defined a "monoterpene mixture", a mixture of 50% cis-ocimene, 17% limonene (90% purity 3:4 ocimene ratio, Sigma-Aldrich®, Bellefonte, USA), and 33% myrcene (90% purity, Sigma-Aldrich®, Bellefonte, USA). We also defined a "benzenoid mixture", a mixture of 66% acetophenone (99.5% purity, Sigma-Aldrich®, Bellefonte, USA), 17% benzaldehyde (99.5% purity, Sigma-Aldrich®, Bellefonte, USA), and 17% methyl benzoate (98%, Sigma-Aldrich®, Bellefonte, USA). We also checked that, during these tests,

the bumblebees were exposed to an olfactory stimulus in the same range as one delivered by real snapdragon inflorescences (results not shown).

Bumblebees were exposed to four different tests. In the first test, bumblebees had to choose between the monoterpene mixture and the monoterpene mixture plus the benzenoid mixture. In the second test, bumblebees were exposed to the monoterpene mixture vs. the monoterpene mixture plus methyl benzoate and benzaldehyde (that is, all benzenoids except acetophenone). In the third test, bumblebees were exposed to the monoterpenes mixture vs. a blank (clean air). In the last test, bumblebees had to choose between acetophenone alone and a blank. Twelve bumblebees were tested in each of these tests. Thus, a total of 48 bumblebees were used in this experiment.

For each bumblebee, we recorded its first choice (i.e., which arm was chosen first) and the proportion of time spent in each arm of the maze during 2 min of observation (time spent in one arm divided by the total time spent in both arms of the maze). Each test was duplicated with the same bumblebee, swapping the presentation side of the olfactory stimuli. Between the observation sessions, the Y-maze was carefully cleaned with an ethanol solution.

Statistical analyses

Differences in VOCs between the blends of the two subspecies were determined based on presence/absence of VOCs and also on the emission rate of each VOC. VOCs that represented less than 0.01% of the total emissions were excluded from the analysis. All statistical analyses were carried out with the R statistical software, version 2.9.2 (http://cran.r-project.org/).

To determine whether some compounds occurred significantly more frequently in one or the other subspecies, a null model of VOC composition was generated for the presence/absence data. Significant difference per VOC was determined using the difference between the randomized sequences of presence/absence generated and the observed difference computed by: $q = (np/Np)-(ns/Ns)$, where, for a given VOC, np is the number of times the VOC was observed in *A. majus pseudomajus* and ns is the number of times it was observed in *A. majus striatum*. The total sample sizes were $Np=40$ in *A. majus pseudomajus* and $Ns=30$ in *A. majus striatum*. Significance was tested at 5% level using a two-way test. If $0<q<0.025$, the VOC was significantly more frequent in *A. majus pseudomajus* than in *A. majus striatum* and if $0.975<q<1$ it was significantly more frequent in *A. majus striatum*.

We used an analysis of variance to test whether the VOC emission intensity was greater in one subspecies than in the other. In this analysis, we included only the floral chemicals

that constituted more than 20% of the total emission of a subspecies to avoid a spurious significance effect due to occasionally emitted VOCs.

For the electroantennogram study (EAG) analyses, the significance of differences of bumblebees' responses was tested using a Mann–Whitney U test at 0.05 level. A Bonferroni correction, as modified by Holm (1979), was computed to adjust alpha of the multiple Mann–Whitney U tests carried out among the bumblebees' responses of the ten VOCs tested in EAG. The classic Bonferroni correction is often considered as being too conservative and Holm's (1979) correction avoids this problem.

For the Y-maze test, a binomial test based on the first choice was computed to determine if the bumblebees exhibit a preference for one of the two volatile chemical signals. Differences in the cumulated time spent in the two arms were tested using a Student's t test. We also tested whether swapping the two same volatile chemical signals between the two arms of the Y-maze yielded the same result for the two recorded variables. To this end, we applied a binomial test for the first choice and a Fisher test for the residence time.

Results

Floral scent composition in the *A. majus* subspecies

The flower scent of the wild snapdragon was composed of a total of 37 VOCs in *A. majus pseudomajus* and 34 VOCs in *A. majus striatum* (Table 1). We detected 20 fatty acid derivatives, including green leaf volatiles, one nitrogen-containing compound (the Syn-3-methyl-butyl-aldoxime), 11 monoterpenes, and five benzenoids.

The two subspecies had a significantly different scent. Three benzenoids (acetophenone, benzaldehyde, and methyl benzoate) were only emitted by *A. majus pseudomajus* and were totally absent in *A. majus striatum* (Fig. 2 and Table 1). This difference in flower scent composition explained the difference in absolute emission rates between *A. majus pseudomajus* and *A. majus striatum* (490 and 150 μg g_{DW}^{-1} h^{-1}, respectively) because acetophenone had the highest emission rate of all VOCs (on average 337 μg g_{DW}^{-1} h^{-1}, Fig. 2 and Table 1). The absolute emission rates of floral VOCs were large, as they exceeded 100 μg g_{DW}^{-1} h^{-1}. Note that we found no correlation between the emission rate and temperature or light intensity. Therefore, we did not correct the measured emission rates for either temperature or irradiance.

In addition to the three benzenoids specific to *A. majus pseudomajus*, eight VOCs were encountered more often in *A. majus pseudomajus* than in *A. majus striatum* (Table 1).

A. majus striatum had no specific VOCs, and only two VOCs were significantly more frequent in *A. majus striatum* than in *A. majus pseudomajus* (ethyl acetate and *p*-cymene, Table 1). Of the six VOCs whose emission rate significantly differed between the two subspecies, two were more abundant in *A. majus striatum* (limonene and γ-terpinene) and four in *A. majus pseudomajus* (6-methyl-5-hepten-2-one and the three specific benzenoids). These results did not depend on the population from which the plants originated.

Bumblebees' responses to olfactory tests

Electroantennography All ten tested VOCs, when used as pure standards, induced a significant antennal electric activity (henceforth depolarization), thus could be detected by the bumblebee antennae (Fig. 3). These VOCs were also detected by antennae when the solutions were diluted at 1%, 10%, and 50%, but not systematically with solutions diluted at 0.1%. In addition, the amplitude of depolarization was maximal and constant with dilutions at 10%, 50%, and pure standards. The three benzenoids, emitted only by *A. majus pseudomajus*, induced a significantly larger maximal depolarization compared with all other VOCs (Fig. 3). In contrast the widespread monoterpenes, ocimene/limonene and myrcene produced small, albeit significant, maximal depolarizations. The three aldehydes, pentanal, hexanal, and nonanal, induced intermediate and highly variable maximal depolarizations (Fig. 3). The compound 2-butanone induced the smallest depolarization in spite of its much higher vapor pressure than that of the three benzenoids (71 mmHg for 2-butanone versus 0.75 mmHg for acetophenone, 0.25 mmHg for methyl benzoate, and 1 mmHg benzaldehyde; all vapor pressures measured at 20° C). Hence, the significantly larger depolarizations recorded in the case of the three benzenoids were not due to higher concentration of these compounds on the antennae. Rather, they were probably caused by a higher number of receptors for these compounds and/or by a higher sensitivity of these receptors.

Spontaneous olfactory preferences in a behavioral assay An analysis of the first choice in the Y-maze showed that there was no difference in performance depending on the side of stimulus presentation, so that data for both tests in which the same volatile chemical signals were swapped were pooled. In the first test comparing monoterpene and monoterpene plus three benzenoids, bumblebees significantly preferred the monoterpene mixture over the monoterpene mixture plus the benzenoid mixture (92%, $Z=2.89$, $p<0.001$, Fig. 4). In the second test, it was the same as the first test, but excluded acetophenone

Table 1 Occurrences (in %) and emission rates (mean and standard error, in μg g_{DW}^{-1} h^{-1}) of floral VOCs in the two *A. majus* subspecies

	A. majus pseudomajus (*n*=40)		*A. majus striatum* (*n*=30)		Test on the occurrences
	Occurrence (%)	Emission rate, μg g_{DW}^{-1} h^{-1}	Occurrence (%)	Emission rate, μg g_{DW}^{-1} h^{-1}	
Fatty acid derivatives		**47.16±15.16**		**45.25±14.57**	
Aldehydes					
2-Methyl-propanal	25	11.31±6.42	47	5.84±2.35	NS
3-Methyl-butanal	33	4.85±2.78	20	2.22±1.57	NS
Pentanal	100	4.60±0.49	100	5.71±0.75	NS
Z-3-Hexenal	65	0.21±0.06	83	0.33±0.11	NS
Hexanal	100	1.33±0.20	100	1.88±0.36	NS
Heptanal	100	0.97±0.17	97	1.19±0.28	NS
Octanal	100	1.45±0.17	83	2.10±0.57	*q*<0.025
Nonanal	100	9.68±1.68	50	7.20±2.99	*q*<0.025
Decanal	58	0.99±0.25	37	2.24±1.17	NS
Alcohol					
1-Pentanol	83	0.58±0.15	50	0.37±0.11	*q*<0.025
Alcenes					
1,3,5-Cycloheptatriene	100	1.31±0.30	100	1.65±0.32	NS
1-Octene	68	0.34±0.11	23	0.17±0.09	*q*<0.025
Alkanes					
1,1-Diethoxy-ethane	53	0.17±0.05	33	0.84±0.41	NS
Nonane	100	0.48±0.13	97	0.84±0.26	NS
Decane	45	0.38±0.15	43	0.22±0.07	NS
Dodecane	38	1.04±0.31	37	1.07±0.51	NS
Esters					
Ethyl acetate	8	0.03±0.02	13	0.20±0.10	*q*>0.975
Hexyl acetate	8	0.11±0.07	20	0.44±0.32	NS
Ketones					
2-Butanone	98	7.26±1.74	100	10.72±2.29	NS
Ether					
Eucalyptol	5	0.07±0.06	3	0.02±0.1	NS
Nitrogen-containing compounds		**1.90±0.38**	**33**	**1.14±0.63**	
syn-3-methyl-butyl-aldoxime	68	1.90±0.38	33	1.14±0.63	*q*<0.025
Monoterpenes		**101.57±13.39**		**103.67±31.14**	
Cyclic					
α-Pinene	58	0.25±0.10	70	0.33±0.07	NS
β-Pinene	30	0.06±0.02	27	0.08±0.04	NS
p-Cymene	8	0.25±0.16	30	0.40±0.18	*q*>0.975
Limonene	95	1.22±0.47	97	2.69±0.69	NS
γ-Terpene	33	0.20±0.10	20	0.45±0.21	NS
Non-cyclic					
β-Myrcene	100	12.82±1.58	100	14.27±3.72	NS
(Z)-β-ocimene	100	1.54±0.49	77	1.47±0.39	*q*<0.025
(E)-β-ocimene	100	78.91±9.22	100	79.99±24.02	NS
3,4-Dimethyl-2,4,6-octatriene	75	3.27±0.63	40	1.95±0.85	*q*<0.025
(E,E)-2,6-dimethyl-1,3,5,7-octatetraene	48	2.10±0.53	20	1.54±0.86	*q*<0.025
Irregulars					
6-Methyl-5-hepten-2-one	100	0.95±0.09	97	0.50±0.11	NS

Table 1 (continued)

	A. majus pseudomajus (n=40)		A. majus striatum (n=30)		Test on the occurrences
	Occurrence (%)	Emission rate, $\mu g g_{DW}^{-1} h^{-1}$	Occurrence (%)	Emission rate, $\mu g g_{DW}^{-1} h^{-1}$	
Benzenoids		**339.86±56.24**		**0.18±0.08**	
Acetophenone	100	337.39±55.44	0	0.00	$q<0.001$
Benzaldehyde	100	0.88±0.08	0	0.00	$q<0.001$
Methyl benzoate	20	1.47±0.65	0	0.00	$q<0.001$
Hemimelitene	15	0.03±0.02	10	0.05±0.04	NS
Mesitylene	15	0.09±0.05	27	0.13±0.04	NS
Total		**490.49**		**150.24**	

The last column shows the significance of a randomized two-tail test on the occurrence of VOCs: if $q<0.025$ the VOC is more frequent in *A. majus pseudomajus* and if $q>0.975$ it is more frequent in *A. majus striatum* (*NS* non-significant test)

of the benzenoid mixture, and the first choice of the bumblebees did not differ significantly between the alternatives (50%, $Z=0.58$, $p>0.05$, Fig. 4). This suggests that acetophenone induces the aversion observed in the first test. In the third test, bumblebees were confronted with the monoterpene mixture vs. a blank (clean air), and they significantly preferred the monoterpene mixture (83%, $Z=2.31$, $p<0.001$, Fig. 4). This result shows that the monoterpene mixture is attractive per se. In the last test, bumblebees were confronted with acetophenone vs. a blank (clean air), and the first choice of the bumblebees did not differ significantly between the alternatives (66%, $Z=0$, $p>0.05$,
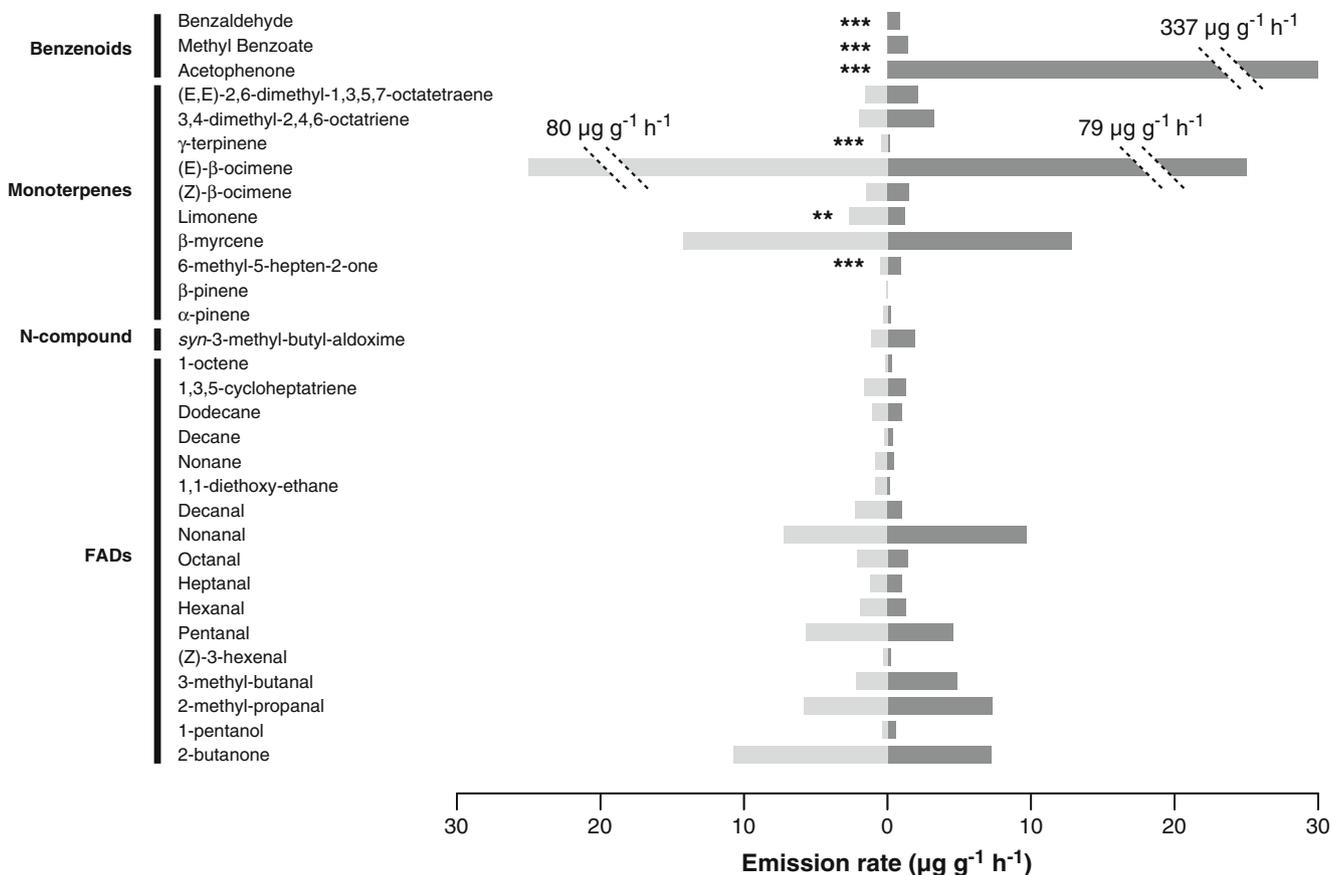

Fig. 2 Mean emission rates ($\mu g g_{DW}^{-1} h^{-1}$) of the floral VOCs emitted by *A. majus pseudomajus* (*dark gray*) and by *A. majus striatum* (*light gray*). *Asterisks* mark the six VOCs for which the emission rate significantly differed between the two subspecies

Fig. 3 Relative amplitude of antennal electric activity (i.e., depolarization) of a total of ten bumblebee antennae for the ten synthetic VOCs analyzed by electroantennography method (EAG). The ten tested VOCs were: *Aceto* Acetophenone, *Benz* Benzaldehyde, *MeBe* Methyl benzoate, *Oci Lim* cis-ocimene and Limonene, *Myrc* Myrcene, *2-But* 2-butanone, *Pent* Pentanal, *Hexa* Hexanal, *Nona* Nonanal. Their respective value of vapor pressure are indicated *between brackets* (mmHg at 20°C, Perry and Green 1997). A pairwise comparison of the signal was performed: *box plots* with the *same letter* at the *top* were not significantly different
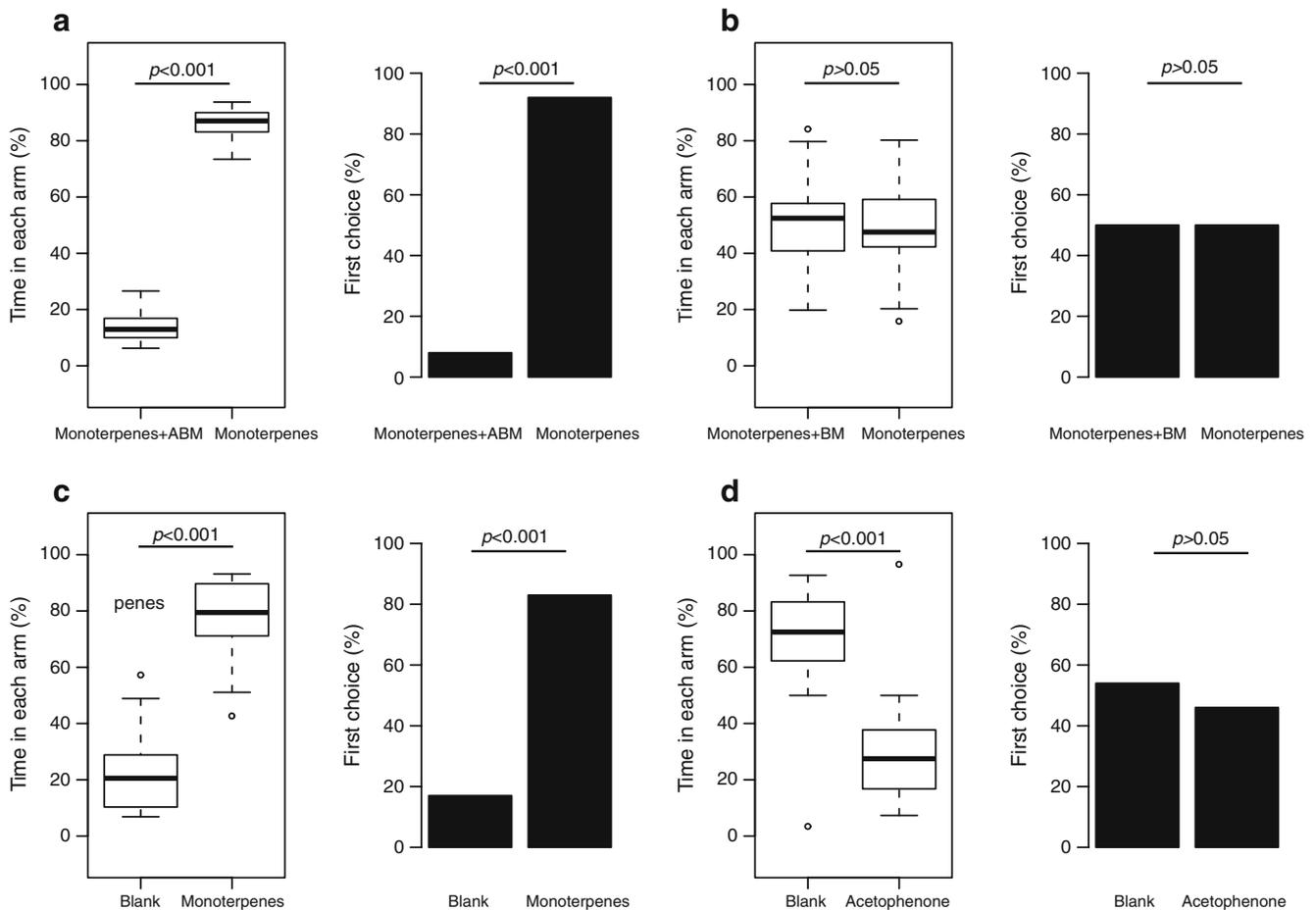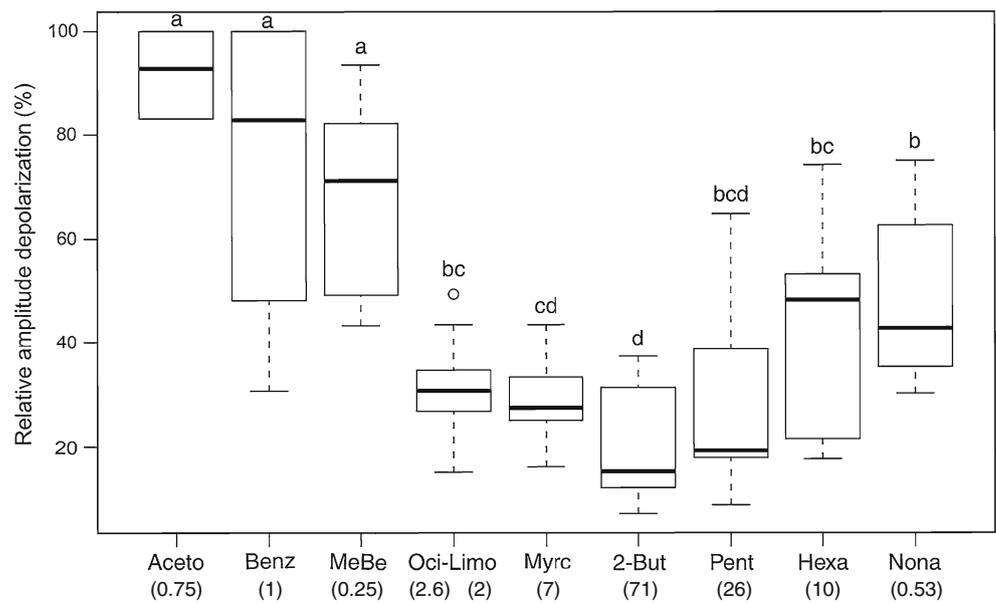
Fig. 4 Percent of time spent by bumblebees in each arm of the Y-maze (*box plots*) excluding the time spent in the entrance and percent of the first choice (*histograms*) for the four pairs of volatile chemical signals tested. **a** First test contrasted three monoterpenes (*cis*-ocimene, limonene, and myrcene, henceforth "monoterpene mixture") to the same monoterpene mixture plus three benzenoids (*A* acetophenone, *B* benzaldehyde, and *M* methyl benzoate; *n*=12) **b** Second test contrasted the monoterpene mixture to the monoterpene mixture plus methyl benzoate and benzaldehyde (i.e., all benzenoids excepted acetophenone; *n*=12) **c** Third test contrasted the monoterpene mixture to a blank (*n*=12); finally, **d** Four tests contrasted acetophenone to a blank (*n*=12)

Fig. 4). This result shows that bumblebees were not repelled by acetophenone but they avoided it; hence, our choice of the term "aversive" instead of "repellent".

An analysis of the time spent in each arm showed that there was no difference in performance depending on the side of stimulus presentation. Hence, data for both tests in which the same volatile chemical signals were swapped, were also pooled for this variable. In the first three tests involving the monoterpene mixture, the time spent in each arm of the maze showed exactly the same trend than the first choice. Bumblebees spent more time in presence of the monoterpene mixture than in presence of the monoterpene mixture plus the benzenoid mixture ($t=44.4$, df=46, $p<0.001$, Fig. 4). They had no preference when acetophenone was removed from this treatment ($t=0.21$, df=46, $p=0.83$, Fig. 4). Finally, they spent more time in presence of the monoterpene mixture than in the 'blank' ($t=13.25$, df=46, $p<0.001$, Fig. 4). The fourth test (acetophenone vs "blank") yielded an interesting new result. Bumblebees spent more time in the "blank" arm than in the arm containing acetophenone ($t=7.26$, df=46, $p<0.001$, Fig. 4), thus confirming the aversive effect of this compound.

Additionally, we quantified the time spent in the main arm of the maze, before entering in one of the arms presenting the olfactory stimuli. This variable reveals the readiness of the bumblebees to choose among stimuli. The decision time did not significantly differ among the first three tests involving monoterpenes, whereas it was significantly different in the last test, where acetophenone alone was emitted ($F=14.64$, df=3, $p<0.001$, Tukey post hoc). Indeed, in the three first tests involving monoterpenes, bumblebees took on average less than 10 s to make a choice (mean and standard error, 4.92 ± 1.44 s; 5.2 ± 1.26 s; 8.2 ± 3.66 s, respectively) whereas they needed three times longer to make a decision in presence of acetophenone alone (27.3 ± 6.32 s).

Discussion

Scent composition of the wild species A. majus

Characterizing the floral scents of *A. majus*, we found that this species emitted up to 37 floral VOCs. In previous studies on horticultural snapdragon, only 12 VOCs were reported (Odell et al. 1999; Wright et al. 2005). In addition, the dominant VOCs of these studies on cultivars were not detected at all in our study (*cis*- and *trans*-methyl-cinnamate, *cis*-3-hexenyl acetate, linalool, dimethoxytoluene, diphenyl ether, and nerolidol). Furthermore, several benzenoids and monoterpenes found to be abundant in our samples were not previously reported (benzaldehyde, hemimelitene, mesitylene, *p*-cymene, α and β-pinene, limonene, γ-terpinene, 6-methyl-5-hepten-2-one, 3,4-dimethyl-1,3,5,7-octatriene and (E,E)-2,6-dimethyl-1,3,5,7-octatetraene). Thus, obvious differences exist between the floral scents of the wild snapdragon and that of the cultivars.

Our second main result was that the two wild subspecies differed strikingly in their flower scent. *A. majus pseudomajus* emitted on average 490 μg g_{DW}^{-1} h^{-1} of flower scent whereas *A. majus striatum* emitted 150 μg g_{DW}^{-1} h^{-1}. All of the *A. majus pseudomajus* populations emitted three benzenoids (acetophenone, benzaldehyde, and methyl benzoate), which were absent from the *A. majus striatum* populations. The benzenoid acetophenone was, by far, the main VOC in *A. majus pseudomajus* but was not detected in *A. majus striatum* (Fig. 2 and Table 1). Such striking differences in floral chemical emissions between subspecies have seldom been reported. Majetic et al. (2007) showed that the floral scent of two sympatric morphs of *Hesperis matronalis* (white and purple flower color) also varied consistently, but they also found a large variation in floral scent of the white morph in the two studied populations. In contrast, the chemical composition of the scents detected in the two *A. majus* subspecies was consistent across populations (C Suchet et al. unpublished data).

Evolution of the floral scent–color association in A. majus

Why do the magenta-flowering phenotypes produce benzenoids, in particular, massive amounts of acetophenone, while the yellow-flowering ones do not? One explanation is that these benzenoids are produced by a biosynthetic pathway related to that of anthocyanin, the flower pigment that produces the magenta coloration. These secondary metabolites may then be jointly regulated. A prerequisite of this scenario would be that the three benzenoids of *A. majus pseudomajus* are indeed synthesized in the same pathway. Benzaldehyde and methyl benzoate are known to be produced in the benzenoid branch of the shikimic acid pathway (Dudareva et al. 2006). Little is known on the biosynthesis of acetophenone, apart that it is reported in 14 of the 90 listed plant families by Knudsen et al. (2006). We suspect that acetophenone is produced in the phenylpropanoid branch of the shikimic acid pathway, as in bacteria (Cripps et al. 1978; Rabus et al. 2002). Further evidence that the biosynthetic pathways of benzenoids and anthocyanins may be linked is offered by the study of Zuker et al. (2002). Indeed, when Zuker et al. (2002) genetically suppressed the expression of a central enzyme of the anthocyanin biosynthesis in transgenic carnation plants (*Dianthus caryophyllus* L.); they observed an over-emission of methyl benzoate. In *A. majus pseudomajus*, both anthocyanins and benzenoids are conspicuously expressed

(as it is genetically known in tobacco, Martin et al. 2001), whereas anthocyanins are only expressed in the vein flower cells of *A. majus striatum* (Schwinn et al. 2006), in which, no benzenoids were detected. Hence, these differences may be explained by differential regulation of the biosynthetic pathways in the two subspecies.

The striking phenotypic difference and scent–color covariation between the two *A. majus* subspecies could also reflect evolutionary responses to multiple selective pressures, including abiotic environment (e.g., based on flower color, Warren and Mackenzie 2001) and the selective pressure of natural predators (Raguso 2009). Field studies that would take into account the local abiotic conditions and the ecological network of interactions are an exciting prospect in the case of the *A. majus* model.

Role of pollinators in maintaining plant species phenotypes

It has been speculated that pollinators are responsible for the maintenance of two distinct phenotypes within *A. majus* (Whibley et al. 2006). Here, we provide new results that shed light on this scenario. We first showed that the two phenotypes have a clearly distinct flower scent. Through EAG analyses, we then showed that the VOCs were detected by the bumblebee antennae and that the three benzenoids emitted by one subspecies but not by the other induced the highest physiological response (Fig. 3). Hence, at least at the antennal level, bumblebees are tuned to detect those VOCs that discriminate between subspecies.

We complemented this analysis by a behavioral experiment, which provided further evidence that bumblebees are innately influenced by VOCs present in the floral scents of *A. majus*. In particular, we showed that bumblebees exhibited an aversion for acetophenone, the most abundant benzenoid, and the one for which the highest depolarization was found in EAG recordings (Fig. 3). Bumblebees were more attracted to the synthetic blend mimicking the *A. majus striatum* flower scent than to that of *A. majus pseudomajus* (Fig. 4a). This preference was not only due to the attractive nature of the monoterpene blend (Fig. 4c) but also to the aversive effect of acetophenone present in *A. majus pseudomajus* (Fig. 4b, d). This is a surprising result because acetophenone was found to be innately attractive to the butterfly *Vanessa indica*, which pollinates plant species in the Asteraceae (Omura and Honda 2005). Despite this difference, our results suggest that VOC emissions should significantly influence the choice behavior of inexperienced *B. terrestris* when foraging in the field.

Such scent-induced discrimination may be enhanced by means of visual cues. We know that the strikingly different colors of the two phenotypes are discriminated by bumblebees (Tastard et al. 2008). Tastard et al. (unpublished results) studied whether *B. terrestris* shows a preference for one of the two colors of scentless artificial snapdragon flowers. They showed that bumblebees preferred the magenta or the yellow color when it was presented against the hybrid colors, but they were not constant in their choice when the magenta and yellow colors were the alternatives. This result tends to support findings of Kunze and Gumbert (2001), which showed that *B. terrestris* discriminate colors more efficiently when the flowers are scented than when they are scentless. Kulahci et al. (2008) also found that bumblebees in the species *Bombus impatiens* trained on flowers differing by their shape and scent learned the rewarding scented flowers faster than those trained on flowers that differed only with respect to visual cues. Hence, the combined action of olfactory and visual cues improves discrimination of the two wild snapdragon subspecies. We emphasize that we did not directly test this hypothesis, but hope to return to this issue in the future.

On the possible ecological role of acetophenone

One open question is why, if *A. majus pseudomajus* emits an aversive scent, it still persists in the wild? A possible explanation could be linked to the flower reward. The nectar of *A. majus pseudomajus* could be of better quality than that of *A. majus striatum*. If so, some pollinators would choose the attractive flower scent (*A. majus striatum*) but gain little reward, while others would learn that the less attractive flower scent also entails a greater reward. Indeed, it has been convincingly shown that bumblebees learn that even compounds that innately induce aversion (e.g., alarm pheromones) may be associated with a reward and thus respond by an appetitive behavior to these compounds (Guerrieri et al. 2005). Variables such as sucrose concentration, amino acid content, alkaloids, etc., may as also be important in the bumblebees' choice (Gegear et al. 2007; Manson et al. 2010). In the future, we plan to quantify the production and the major constituents of the nectar to test whether or not *majus pseudomajus* flowers compensate the aversive effect of acetophenone emission by offering richer or less deterrent nectar to pollinators.

Acetophenone may also act as a defensive compound. Indeed, acetophenone has been shown to deter the western pine wood-boring beetle *Dendroctonus brevicomis* (Erbilgin et al. 2008). The effect of acetophenone could be tested in other insects interacting with *A. majus*. Hence, acetophenone may potentially repel herbivores such as the weevil *Rhinusa vestita* (that lays its eggs into the fertilized ovaries) or the caterpillars of the butterfly *Mellicta deione* (that feeds exclusively on wild snapdragon leaves, C. Thébaud pers. comm.). Acetophenone would be clearly advantageous as a deterrent compound of *R. vestita*, as this weevil may use flower scent to localize its nursery flowers. Preliminary tests showed that snapdragon leaves do not emit acetophenone. If

M. deione is deterred by floral acetophenone, this would explain why some plants are entirely defoliated but still maintain their flowers.

In conclusion, we have shown that wild *A. majus* differ from *A. majus* cultivars in the composition of flower scent. *A. majus pseudomajus* and *A. majus striatum*, the two natural subspecies of *A. majus*, emitted different scents, which were discriminated by naive floral visitor such as *B. terrestris*. Bumblebees innately avoided acetophenone, the main VOC in *A. majus pseudomajus*, absent from the floral scent of *A. majus striatum*. These findings point to a crucial role of floral VOCs in the evolutionary ecology of *A. majus*.

Appendix 1: Flower visitor census

We performed a flower visitor census of the two studied *A. majus* subspecies between May and June 2009 for a total of 4,000 min of observation (500 min in eight populations). Each insect that was observed to enter a flower was caught. Taxonomic determinations were kindly provided by Prof. Pierre Rasmont (Université de Mons, Belgium).

We observed a total of 118 pollinators (see the table below). The frequency of visit was low: about two pollinators per hour in a patch of at least 50 flowers. Over 65% of the pollinators belonged to the genus *Bombus* (10 species/subspecies), 18% to the tribe *Halictini*, 12% *Xylocopa violacea*. The rest included a range of infrequent species resumed in the following table:

Visitor species	n
B. terrestris lusitanicus Krüger	14
B. terrestris dalmatinus Dalla Torre	5
B. terrestris terrestris (L.)	1
B. hortorum (L.)	13
B. ruderatus ruderatus (Fabricius)	2
B. ruderatus autumnalis (Fabricius)	1
B. humilis quasimuscorum Vogt	3
B. pratorum (L.)	2
B. rupestris vasco (Lepeletier)	1
B. sylvarum (L.)	1
Bombus sp. (not trapped)	34
Tribe *Halictini*	21
Xylocopa violacea (L.)	14
Rhodanthidium sticticum (Fabricius)	3
Anthophora mucida Gribodo	1
A. aestivalis (Panzer)	1
Hylaeus sp.	1
Total	118

References

Adams RP (2001) Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. Publishing corporation, Carol Stream, Illinois, Allured

Andalo C, Cruzan MB, Cazettes C, Pujol B, Burrus M, Thébaud C (2010) Post-pollination barriers do not explain the persistence of two distinct *Antirrhinum* subspecies with parapatric distribution. Plant Syst Evol 286:223–234

Chittka L, Thomson JD (2001) Cognitive ecology of pollination: animal behaviour and floral evolution. Cambridge University, Cambridge

Comba L, Corbet SA, Hunt H, Outram S, Parker JS, Glover BJ (2000) The role of genes influencing the corolla in pollination of *Antirrhinum majus*. Plant Cell Environ 23:639–647

Cripps RE, Trudgill PW, Whateley JG (1978) The metabolism of 1-phenylethanol and acetophenone by *Nocardia* T5 and an *Arthrobacter* species. Eur J Biochem 86:175–186

Deisig N, Lachnit H, Giurfa M (2002) The effect of similarity between elemental stimuli and compounds in olfactory patterning discriminations. Learn Mem 9:112–121

Dudareva N, Murfitt LM, Mann CJ, Gorenstein N, Kolosova N, Kish CM, Bonham C, Wood K (2000) Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. P Cell 12:949–961

Dudareva N, Martin D, Kish CM, Kolosova N, Gorenstein N, Fäldt J, Miller B, Bohlmann J (2003) (E)-β-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily. P Cell 15:1227–1241

Dudareva N, Negre F, Nagegowda DA, Orlova I (2006) Plant volatiles: recent advances and future perspectives. Crit Rev Plant Sci 25:417–440

Dupuy F, Sandoz J-C, Giurfa M, Josens R (2006) Individual olfactory learning in *Camponotus* ants. Anim Behav 72:1081–1091

Erbilgin N, Gillette NE, Owen DR, Mori SR, Nelson AS, Uzoh F, Wood DL (2008) Acetophenone superior to verbenone for reducing attraction of western pine beetle *Dendroctonus brevicomis* to its aggregation pheromone. Agric For Entomol 10:433–441

Gegear RJ, Manson JS, Thomson JD (2007) Ecological context influences pollinator deterrence by alkaloids in floral nectar. Ecol Lett 10:375–382

Giurfa M (2007) Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. J Comp Physiol A 193:801–824

Giurfa M, Menzel R (1997) Insect visual perception: complex abilities of simple nervous systems. Curr Op Neurobio 7:505–513

Giurfa M, Vorobyev M (1997) The detection and recognition of color stimuli by honeybees: performance and mechanisms. Isr J Pl Sci 45:129–140

Goodwin SM, Kolosova N, Kish CM, Wood KV, Dudareva N, Jenks MA (2003) Cuticle characteristics and volatile emission of petals in Antirrhinum majus. Physiol Plant 117:435–443

Guenther A, Hewitt CN, Erickson D, Fall R, Geron C, Graedel T, Harley P, Klinger L, Lerdau M, Mckay WA, Pierce T, Scholes B, Steinbrecher R, Tallamraju R, Taylor J, Zimmerman P (1995) A global model of natural volatile organic compound emissions. J Geophys Res 100:8873–8892

Guerrieri F, Schubert M, Sandoz J-C, Giurfa M (2005) Perceptual and neural olfactory similarity in honeybees. PLoS Biol 3:718–732

Harder LD, Barrett SCH (2006) Ecology and evolution of flowers. Oxford University, Oxford

Heinrich B, Raven PH (1972) Energetics and pollination ecology. Science 176:597–602

Holm S (1979) A simple sequentially rejective multiple test procedure. Scand J Stat 6:65–70

Jorgensen AD, Picel KC, Stamoudis VC (1990) Prediction of gas chromatography flame ionization detector response factors from molecular structures. Anal Chem 62:683–689

Kesselmeier J, Schäfer L, Ciccioli P, Brancaleoni E, Cecinato A, Frattoni M, Foster P, Jacob V, Denis J, Fugit JL, Dutaur L, Torres L (1996) Emission of monoterpenes and isoprene from a Mediterranean oak species Quercus ilex L. measured within the BEMA (Biogenic Emissions in the Mediterranean Area) project. Atmos Environ 30:1841–1850

Knudsen JT, Eriksson R, Gershenzon J, Stahl B (2006) Diversity and distribution of floral scent. Bot Rev 72:1–120

Komenda M, Parusel E, Wedel A, Koppmann R (2001) Measurements of biogenic VOC emissions: sampling, analysis and calibration. Atmos Environ 35:2069–2080

Komischke B, Giurfa M, Lachnit H, Malun D (2002) Successive olfactory reversal learning in honeybees. Learn Mem 9:122–129

Kunze J, Gumbert A (2001) The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. Behav Ecol 12:447–456

Kulahci IG, Dornhaus A, Papaj DR (2008) Multimodal signals enhance decision making in foraging bumble-bees. Proc R Soc B 275:797–802

Majetic C, Raguso R, Tonsor S, Ashman T (2007) Flower color-flower scent associations in polymorphic Hesperis matronalis (Brassicaceae). Phytochemistry 68:865–874

Manson JS, Otterstatter MC, Thomson JD (2010) Consumption of a nectar alkaloid reduces pathogen load in bumble bees. Oecologia 162:81–89

Martin C, Jin H, Schwinn K (2001) Mechanisms and applications of transcriptional control of phenylpropanoid metabolism. In: Colney N (ed) Regulation of phytochemicals by molecular techniques. Elsevier Science Ltd, Oxford, pp 155–170

Mitchell RJ, Irwin RE, Flanagan RJ, Karron JD (2009) Ecology and evolution of plant-pollinator interactions. Ann Bot 103:1355–1363

Moukhtar S, Bessagnet B, Rouil L, Simon V (2005) Monoterpene emissions from Beech (Fagus sylvatica) in a French forest and impact on secondary pollutants formation at regional scale. Atmos Environ 39:3535–3547

Odell E, Raguso RA, Jones KN (1999) Bumblebee foraging responses to variation in floral scent and color in snapdragons (Antirrhinum: Scrophulariaceae). Am Midl Nat 142:257–265

Omura H, Honda K (2005) Priority of color over scent during flower visitation by adult Vanessa indica butterflies. Oecologia 142:588–596

Perry RH, Green DW (1997) Perry's chemical engineers' handbook. Mc Graw-Hill, New York

Rabus R, Kube M, Beck A, Widdel F, Reinhardt R (2002) Genes involved in the anaerobic degradation of ethylbenzene in a denitrifying bacterium, strain EbN1. Arch Microbiol 178:506–516

Raguso RA (2008) Wake up and smell the roses: the ecology and evolution of floral scent. Annu Rev Ecol Evol Syst 39:549–569

Raguso RA (2009) Floral scent in a whole-plant context: moving beyond pollinator attraction. Funct Ecol 23:837–840

Ricklefs RE, Renner SS (1994) Species richness within families of flowering plants. Evolution 48:1619–1636

Riffell JA, Alarcon R, Abrell L, Davidowitz G, Bronstein JL, Hildebrand JG (2008) Behavioral consequences of innate preferences and olfactory learning in hawkmoth-flower interactions. Proc Natl Acad Sci 105:3404–3409

Roelofs WL (1984) Electroantennogram assay: rapid and convenient screening procedures for pheromones. In: Hummel HE, Miller TA (Eds.). Techniques in pheromone research US, Springer Verlag, pp 131–160

Sabillon D, Cremades L (2001) Diurnal and seasonal variation of monoterpene emission rates for two typical Mediterranean species (Pinus pinea and Quercus ilex) from field measurements-relationship with temperature and PAR. Atmos Environ 35:4419–4431

Schatz B, Djieto-Lordon C, Dormont L, Bessière J-M, McKey D, Blatrix R (2009) A simple, non-specific chemical signal mediates defence behaviour in a specialised ant-plant mutualism. Curr Bio 19:361–362

Schwinn K, Venail J, Shang Y, Mackay S, Alm V, Butelli E, Oyama R, Bailey P, Davis K, Martin C (2006) A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus Antirrhinum. P Cell 18:831–851

Simon V, Dumergues L, Solignac G, Torres L (2005a) Biogenic emissions from Pinus halepensis: a typical species of the Mediterranean area. Atmos Res 74:37–48

Simon V, Dumergues L, Bouchou P, Torres L, Lopez A (2005b) Isoprene emission rates and fluxes measured above a Mediterranean oak (Quercus pubescens) forest. Atmos Res 74:49–63

Stebbins L (1970) Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms. Annu Rev Ecol Syst 1:307–326

Tastard E, Andalo C, Giurfa M, Burrus M, Thébaud C (2008) Flower colour variation across a hybrid zone in Antirrhinum as perceived by bumblebee pollinators. Arthropod Plant Interact 2:237–246

Tholl D, Boland W, Hansel A, Loreto F, Röse USR, Schnitzler J-P (2006) Practical approaches to plant volatile analysis. Plant J 45:540–560

Thiéry D, Marion-Poll F (1998) Electroantennogram responses of Douglas-fir seed chalcids to plant volatiles. J Insect Physio 44:483–490

Warren J, Mackenzie S (2001) Why are all colour combinations not equally represented as flower-colour polymorphisms? New Phytol 151:237–241

Whibley AC, Langlade NB, Andalo C, Hanna AI, Bangham A, Thébaud C, Coen E (2006) Evolutionary paths underlying flower color variation in Antirrhinum. Science 313:963–966

Wright GA, Lutmerding A, Dudareva N, Smith BH (2005) Intensity and the ratios of compounds in the scent of snapdragon flowers affect scent discrimination by honeybees (Apis mellifera). J Comp Physiol A 191:105–114

Zuker A, Tzfira T, Ben-Meir H, Ovadis M, Shklarman E, Itzhaki H, Forkmann G, Martens S, Neta-Sharir I, Weiss D, Vainstein A (2002) Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. Mol Breed 9:33–41