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REGULAR PAPER

Relationships among floral VOC emissions, floral rewards and visits of pollinators in five plant species of a Mediterranean shrubland

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Background and aims – In plant-pollinator communities seasonal changes in the abundance of pollinators lead to seasonal changes in competition among flowering plants for their services. Here we address the following question: Do flowers of a given species produce more olfactory signals (emissions of volatile compounds) and rewards (nectar and pollen) during the phase(s) of the flowering period within which they have to maximally compete with the signals and rewards of other co-flowering species in the community, compared to the amount of signals and rewards produced during the period(s) with less floral competition?
Methods – We analysed the floral emission rates of biogenic volatile organic compounds by gas chromatography and proton transfer reaction mass spectrometry, the visitation rates of pollinators, and the availability of nectar and pollen during the flowering periods of five species to test whether floral rewards and signals would decrease with an increase in pollinator visitation rates during late spring and early summer, i.e. coinciding with decreasing competitive pressure for the services of pollinators.
Key results – The results indicate that phenological patterns in the production of rewards are only present at the species level in those species with long flowering periods or with matching periods of changes in pollinator populations. The capacity of emitting isoprenoids and oxidised volatile organic compounds, however, did not present significant patterns during the flowering period in any of the five species studied.
Conclusions – The results support the hypothesis of a decreasing competitive pressure for the attraction of pollinators that may drive a decrease in floral investment in rewards but not an accompanying decrease of the capacity of emitting volatile olfactory signals in a species with long flowering period. However, the negative correlation between nectar production and visitation rates may be reinforced by the opposite responses of these variables to climatic conditions. This fact makes difficult to discern possible evolutionary forces tending to decrease rewards from plastic responses to changing environmental conditions in that part of the flowering period in which pollinator visitation rates are higher.

Key words – Plant-pollinator interaction, biological market, floral scent, floral phenology, *Rosmarinus officinalis*, *Muscari neglectum*, *Euphorbia flavicoma*, *Biscutella laevigata*, *Phlomis lychnitis*.

INTRODUCTION

Plants produce and emit a great diversity and large amounts of biogenic volatile organic compounds (BVOCs), which are considered predominantly secondary products of plant metabolism (Knudsen et al. 2006). BVOCs have significant biological and environmental effects on the relationships of plants with other organisms (Dudareva et al. 2006) and on the chemistry and physics of the atmosphere (Peñuelas & Staudt 2010). These BVOC emissions serve different functions in plants: protection against extreme environmental conditions (Sharkey & Singsaas 1995, Peñuelas & Llusia 2003, Peñuelas & Munné-Bosch 2005, Niinemets 2009); deterrence of detrimental organisms such as herbivores (Peñuelas et al. 2005a, Piesik et al. 2010); attraction of beneficial organisms such as pollinators, seed dispersers or predators and parasitoids of herbivores (Pichersky & Gershenzon 2002, Filella et al. 2011); attraction of insect preys, in the case of carnivorous plants (Di Giusto et al. 2010); identification of plant competitors in the vicinity by the detection of their BVOC cues (Kegge & Pierik 2010, Seco et al. 2011); inhibition of some biological processes (alellopathy) of nearby competitors (Peñuelas et al. 1996, Kegge & Pierik 2010); and communication between individuals of the same species, between different species, and between different tissues of the same plant (Piesik et al. 2010, Seco et al. 2011).

Most of these BVOC functions are still poorly understood and require more investigation. One function that specially warrants investigation is the use of BVOCs as signals for the communication between plants and their pollinators (Farré-Armengol et al. 2013), particularly the intricate relationship of signals between plants and pollinators in diverse plant-pollinator communities (Kessler & Halitschke 2009, Vázquez et al. 2009).

Flowers present rewards to attract pollinators. The main rewards are nectar and pollen. Nectar is a sugar-rich liquid whose production in flowers is highly related to the energy requirements of pollinator species, especially when flowers are pollinated by only one or a few species (Heinrich & Raven 1972). The investment of resources into the production of nectar is so important in some plant species that they have evolved a variety of mechanisms to exclude nectar 'thieves', those visitors to flowers that consume nectar but are inefficient pollinators (Irwin et al. 2004). Pollen also acts as a floral reward, especially to bees, one of the most ubiquitous and important groups of pollinators that can range from generalists to specialists. Pollen is used by bees as a source of protein (Roulston & Cane 2000).

To benefit from such rewards, though, pollinators must be able to recognise flowers. Plants thus have a diverse array of traits to attract pollinators, within which visual characteristics and scents of flowers play key roles (Chittka & Raine 2006). Thousands of plant species pollinated by insects actively emit specific signals of floral scents, even though the production and emission of these volatile molecules are both metabolically costly (Vogel 1983) and risky, as they may attract unwanted visitors such as herbivores (Baldwin et al. 1997). The investment in scent production as an advertisement of reward, though, can improve plant fitness (Majetic et al. 2009).

Plant-pollinator systems consist of complex networks that can be considered as a biological market in which pollinators are exposed to a diverse array of flower species, among which they choose those with the best rewards (Chittka & Raine 2006). Plants must attract and sometimes compete for the attention and services of pollinators. The distribution of visitors among flowers is strongly affected by competition and facilitation occurring between plants (Ghazoul 2006), and by competition between pollinators for the exploitation of floral resources (Pleasants 1981). Many pollinators learn the particular scent signals of different species to recognize those flowers offering the highest quality rewards (Chittka et al. 1999). The olfactory sensory acuity of, and olfactory learning by pollinators thus have a strong effect on the evolution of floral signals, due to their effect on the selective forces exerted by pollinators through their impact on plant fitness (Wright & Schiestl 2009). However, not all floral rewards are available for all the potential visitors in a community. Some plant species present physical barriers or chemical filters that restrict the access to floral rewards by some pollinators of the community and serve to avoid their consumption by thieves (Johnson & Steiner 2000, Shuttleworth & Johnson 2009).

Floral structures such as petals, sepals, and stamens emit volatile substances for attracting pollinators (Dötterl & Jürgens 2005, Mena et al. 2005, Flamini et al. 2007). While some floral volatiles are specific attractants of particular insect species (e.g. Eltz et al. 1999, Schiestl et al. 2003), others are common BVOCs that become attractive for a large array of generalist pollinators (e.g. Li et al. 2008, Johnson & Hobbhahn 2010). All these compounds act as chemical cues that facilitate floral location by creating concentration gradients that pollinators perceive with their sensory receptors (Chittka & Raine 2006). In some cases, concentration gradients of BVOCs also indicate the route to floral nectaries (Pichersky & Gershenzon 2002, Dötterl et al. 2012), the floral structures that contain nectar, and visitors then pollinate flowers by accidentally carrying pollen from one flower to another during their search for nectar. Terpenoid emissions have been described to play attractive functions in flowers, contrasting with their basically defensive functions in leaves and other vegetative plant parts (Farré-Armengol et al. 2013). Moreover, terpenoids have been suggested to be major contributors to the effect of floral scent emissions on seed fitness (Majetic et al. 2009).

In most Mediterranean entomophilous plant communities, flowering peaks in early spring (March-April), while the peak of abundance of the majority of pollinators occurs in late spring and summer (Petanidou et al. 1995, Bosch et al. 1997). The spring maximum of flowering causes an excess of flowers in relation to the abundance of pollinators, and in response, an intense competition between plants for the attention of pollinators arises, which is biologically reflected in a large investment in rewards (pollen and nectar) and cues for identification and location (visual and olfactory) in those species flowering only or mainly during this phase of the season (Cohen & Shmida 1993). In late spring and summer, the situation is reversed; a surplus of insects over flowers occurs, so that a reduction of floral investment in attraction would be expected (Shmida & Dafni 1989). This scenario is plausible because biotic interactions have the potential to influence aspects of the flowering phenology of plants (Elzinga et al. 2007).

In a recent study, Filella et al. (2013) conducted a series of measurements in a Mediterranean coastal shrubland community, and found that floral volatile emissions were highest in the species flowering during the first months of spring. The flowers presented maximal rewards when pollinator visitation rates were at a minimum. Volatile emissions were lowest in those species flowering in late spring-early summer when the availability of rewards was lower and pollinator visitation rates were at a maximum. These relationships are of great interest for the resource economy of plants, which is strongly influenced by the large investment in floral resources that most plants assume during their blooming periods. A possible reduction in investment in floral signals and rewards in the final stage of the community's peak flowering period by spring- and summer-blooming species (when many pollinators have fewer floral resources available to them) can lead to a considerable saving of resources (Gershenzon 1994) without implying a decrease in plant fitness, because pollinators are more abundant and active and fewer plant species in flower are competing for the services of pollinators. Here, we addressed this question at the intraspecific level, i.e. we aimed to determine whether floral BVOC emissions and floral rewards (pollen and nectar) decrease along the flowering

period of each single species, coinciding with the described seasonal pattern of increasing abundance and activity of pollinators and decreasing numbers of coexisting plant species in flower. Our hypothesis assumes that this would be advantageous for the plant to maximize the investment in flower rewards and signals when there is maximal competition for pollinators, and reduce this investment when competition decreases to save a significant amount of resources.We tested these possible patterns in five plant species encompassing a range of flowering periods: early spring (*Rosmarinus officinalis* L. and *Muscari neglectum* Guss.), late spring (*Euphorbia flavicoma* DC. and *Phlomis lychnitis* L.), or throughout the entire spring period (*Biscutella laevigata* L.).

MATERIALS AND METHODS

Study area and sampling design

Field work was performed at Garraf Natural Park on the central coast of Catalonia (NE Spain) in 2011. The experimental zone was located at 400 m a.s.l. and 2800 m from the coast (UTM: 31T, 408256 m, 4570749 m). The climate is typically Mediterranean and is strongly influenced by proximity to the sea, with sparse but torrential rain during spring and autumn, temperate winters, and hot and dry summers. The plant community in the sampling zone is a shrubland dominated by Kermes oak (*Quercus coccifera* L.) and mastic tree (*Pistacia lentiscus* L.), with dwarf fan palm (*Chamaerops humilis* L.), Mauritania vine reed (*Ampelodesmos mauritanica* (Poir.) T.Durand & Schinz), Killarney strawberry tree (*Arbutus unedo* L.), Mediterranean buckthorn (*Rhamnus alaternus* L.), rosemary (*Rosmarinus officinalis*), Mediterranean heath (*Erica multiflora* L.), Salvia cistus (*Cistus salviifolius* L.), and a large variety of geophytes (*Muscari neglectum*, *Gladiolus illyricus* W.D.J. Koch, *Ranunculus* sp. L., *Anacamptys piramidalis* (L.) Rich., and *Narcissus assoanus* Dufour ex Schult. & Schult.f.) and dwarf shrubs (*Helianthemum* sp., *Euphorbia flavicoma*, *Polygala rupestris* Pourr., *Biscutella laevigata*, and *Phlomis lychnitis*). The community of pollinators and plants present in this area and their relationships are described in detail in Bosch et al. (2009).

Five individuals each of *R. officinalis*, *M. neglectum*, *B. laevigata*, *E. flavicoma*, and *P. lychnitis* were randomly selected from a reduced area (less than one hectare) to minimise the effects of local variability in microclimate. Once a week, we (1) counted the number of visits of pollinators to the individual plants and the number of open flowers per individual, (2) measured floral nectar production, (3) harvested undehisced anthers for measuring pollen production in the laboratory, and (4) collected flowers for analysing BVOC emissions under standard conditions in the laboratory. Five samples for analysis by gas chromatography mass spectrometry (GC-MS) and five for analysis by proton transfer reaction mass spectrometry (PTR-MS) were collected each week from the beginning of flowering (11 March for the earliest flowering) to the end of flowering (16 June for the latest flowering). We conducted all measurements from the same individuals, with the exception of those of *M. neglectum*. In this species, we used ten different individuals each week because *M. neglectum* produces only one small inflorescence per individual during its flowering period, so we sampled the entire inflorescences to have enough material for the BVOC analyses. Measurements of nectar, pollen, and pollinator visits were only conducted on the five individuals employed for the GC-MS analyses in *M. neglectum*.

Pollinator visitation rate

Pollinator visits were always counted on sunny days between 9:30 h and 13:30 h, because the activity of insects is strongly correlated with temperature. A count consisted on annotating all insects that visited the plant individual during a four-minute interval. For visitation counts, one person stopped in front of the plant individual whose visits were going to be counted, but always at a certain distance to avoid interferences on insect behaviour. A visit was recorded when an insect stopped on at least one flower to feed on nectar or collect pollen from the individual plant. Consecutive visits made by an insect individual to different flowers of the same plant individual were counted only as one visit. But when an insect left the plant individual that was being observed to visit flowers from another plant individual and then returned to visit flowers of the observed one, two different visits were recorded. We identified insects to the level of order. For hymenopterans, the insect order that interacted most with flowers, we also distinguished among bees, bumblebees, wasps, and ants. We later excluded the recorded flower visitors that were not efficient at pollinating flowers and may have exerted a neutral or negative effect on plant fitness. The counts were repeated usually ten times (at least six times) in the same week for each of the five individuals of each plant species to obtain a better estimate of the abundance of pollinators visiting flowers for each individual and week. The total number of open flowers was counted for each of the studied individuals every day we conducted visitation counts. Rates of visitation were finally converted to number of visits per 100 flowers per hour.

Nectar and pollen production

To avoid underestimating nectar production, we covered the flowering stems and inflorescences with fine-mesh gauze bags the day before the measurement, trying to avoid any modification of nectar production by affecting the microenvironment (Wyatt et al. 1992). We thereby excluded insects and prevented them from consuming the nectar during the 24 hours before measuring. The nectar accumulated was extracted with micropipettes (0.25, 0.5, and 1 ml). These measurements were made once a week for five flowers of each of the five individuals of each species.

To assess pollen production, we harvested undehisced anthers of randomly selected flowers and preserved them in vials containing 300 µl of 70% ethyl alcohol. We opened the anthers inside the vial with a needle under a microscope in the laboratory and used an ultrasonic sonicator to completely empty the pollen contents and to separate the pollen aggregates. The vial was then vortexed to dilute and homogeneously distribute the pollen in suspension. A known volume of the vial contents was added to a microscope slide with a 0.1 µl counting chamber to count the number of pollen grains per anther. Six subsamples per sample were counted, and the av-

erage calculated. The total pollen production per flower was then calculated for the total number of anthers per flower.

BVOC emission rates

The flowers or inflorescence stems were cut and immediately recut under water and put into small glass bottles filled with water. The samples were collected at midday and immediately carried to the laboratory in a refrigerator at 4°C. At the laboratory, the BVOC emissions from samples were analyzed by both static and dynamic headspace techniques with GC-MS and PTR-MS, respectively. The PTR-MS measurements with a dynamic headspace method provided emission rates of monoterpenes, sesquiterpenes, isoprene, and oxygenated short-chain BVOCs. The GC-MS static headspace measurements provided the ratios among different terpenoids, and this allowed us to convert the total mono- and sesquiterpene emission rates from PTR-MS dynamic headspace measurements into the emission rates of each single compound.

Terpene analyses were performed by GC-MS (Agilent Technologies, GC: 7890A, MS: 5975C inert MSD with Triple-Axis Detector, Palo Alto, CA, USA). The flowers were placed in 10mL vials in a Head Space incubator (CTC Analytics, MH 01-00B, Zwingen, Switzerland) and later processed with an automatic sample processor (Combi PAL, CTC Analytics, MXY 02-01B, Zwingen, Switzerland). For *M. neglectum* we used an entire inflorescence per sample, while for the other four species we used 2–3 flowers per sample. Incubation time was 1 min at 35°C. Using a Head Space 2.5 mL syringe (CTC Analytics, MSH 02-00B, Zwingen, Switzerland), 2 mL samples were injected into a capillary column of 30 m × 0.25 mm × 0.25 mm (HP-5MS, Agilent Technologies). Helium flow was 1ml min^{-1}. Total run time was 26 min. After sample injection, the initial time was 1 min, and the initial temperature (35°C) was then increased at 15ºC min^{-1} to 150°C and maintained for 5 min, at 50°C min^{-1} to 250ºC and maintained for 5 min, and finally at 30°C min^{-1} to 280°C and maintained for 5 min.

Monoterpenes were identified by comparing the retention times with liquid standards from Fluka (Buchs, Switzerland) volatilised in vials and the fractionation mass spectra with standard spectra and Nist05a and wiley7n mass spectra libraries. Terpene concentrations were determined from calibration curves. The calibration curves for the common monoterpenes α-pinene, β-pinene, 3-carene, and linalool and common sesquiterpenes such as α -humulene were determined once every seven analyses. Terpene calibration curves (n = 4 different terpene concentrations) were always highly significant ($r^2 > 0.99$) in the relationship between signal and terpene emission rates.

Simultaneously with the GC-MS measurements, other floral samples (one inflorescence for *M. neglectum* and one or two flowers for the other four species) from the same individual plants were clamped in a 90 cm^3 PLC-2 ADC cuvette connected to an infrared gas analyser (LCA-4, ADC; Hoddeson, Hertfordshire, UK). BVOC-free zero air was fluxed into the cuvette, and the exiting air was sent to a PTR-MS system. The cuvette was maintained at 30°C and 1000 µmol m^{-2} s^{-1} PAR. Because the flow through the cuvette (about 250 mL/min) was higher than the flow needed for PTR-MS (50–100mL/min), part of the flow was channelled through an overflow outlet. The cuvette was lined with Teflon, and only Teflon tubing, connectors, and valves were used, to reduce the surface interactions in the system. The measurements of gas exchange were also conducted with an empty cuvette as an additional control. Part of the air exiting the leaf cuvette thus flowed through a T-system to the PTR-MS inlet. The PTR-MS is a highly sensitive device (PTR-MS-FTD hs; Ionicon Analytik, Innsbruck, Austria) consisting of three parts: the ion source, where ions are produced by a hollow cathode discharge using water vapour as the molecular source of ions; the drift tube, where proton-transfer reactions to the trace constituents in the air occur (BVOCs with a higher proton affinity than that of water (166.5 kcal mol^{-1}), including most unsaturated and almost all oxygenated hydrocarbons, undergo a proton-transfer reaction with H_3O^+); and the ion detector, which provides sensitive detection of the mass-selected ions that are characteristic of the molecules of interest. Both the PTR-MS and its use in BVOC analysis have been described in detail elsewhere (Lindinger et al. 1998, Peñuelas et al. 2005b). Here, the PTR-MS drift tube was operated at 2.1 mbar and 40°C, with a drift field of 600 V cm^{-1}. The parent ion signal was maintained at ca. 2×10^6 counts s^{-1} during the measurements. We conducted scans of all masses between 41 and 206.

For the determination and quantification of BVOC exchange, the air both entering and exiting the cuvette was monitored with flow meters and analysed with PTR-MS (Ionicon Analytik, Innsbruck, Austria) at alternate intervals. The difference between the concentrations of BVOCs before and after passing through the chambers, along with the flow rates, was used to calculate the BVOC exchange. The tubing used to connect the cuvette to the PTR-MS system was made of Siltek-passivated stainless steel (Restek, Bellefonte, PA, USA).

Statistical treatment

We used STATISTICA 8 for testing the existence of seasonal patterns of change in our phenological variables. We checked and confirmed that the data presented normal distribution of the residuals and heteroscedasticity. We conducted general linear models with the Julian date as the explanatory variable and each of our measured variables as the response variable, while including the individuals as a random factor in the models of those species in which we conducted repeated measures on the same individuals at different weeks (all the species with the exception of *M. neglectum*). We further analyzed the relation of rewards and emissions with visitation rates, by conducting general linear models with visitation rates as the explanatory variable and rewards and emissions as the response variables, while including the individuals as a random factor. Finally, we conducted a multivariate analysis for *B. laevigata* that consisted of a generalized linear mixed model with pollinator visitation rates as the response variable, pollen and nectar productions, terpene emission capacities and mean temperature of the day as explanatory variables, and individuals as a random factor.

RESULTS

Characterisation of flowering phenology, rewards, volatile emissions, and visits

Muscari neglectum and *R. officinalis* flowered from late winter to early spring, *E. flavicoma* from early to mid-spring, *B. laevigata* from early to late spring (the longest flowering period of the five species studied), and *P. lychnitis* in late spring (fig. 1A). *Phlomis lychnitis* and *R. officinalis* produced the most nectar per flower, *M. neglectum* produced less, and *B. laevigata* and *E. flavicoma* produced very little nectar (fig. 1B). *Phlomis lychnitis* produced the most pollen, followed by *M. neglectum*, *B. laevigata*, *R. officinalis*, and *E. flavicoma* (fig. 1C). *Rosmarinus officinalis* had by far the the highest rates of terpene emissions (fig. 2) and the highest variability of compounds identified. *Muscari neglectum* and *P. lychnitis* had very low rates of terpene emissions, followed by *B. laevigata* and *E. flavicoma*, which had the lowest rates of terpene emissions. The flowers of *R. officinalis* emitted α-pinene, camphene, β-pinene, and camphor (fig. 2). They also emitted small amounts of eucalyptol, α-phellandrene, γ-terpinene, α-terpinolene, and isoborneol. *Muscari neglectum* emitted α-pinene, trans-β-ocimene, acetophenone, and trans-β-cayophyllene. *Biscutella laevigata* only emitted detectable amounts of trans-β-ocimene. *Euphorbia flavicoma* emitted trans-β-ocimene and trans-β-caryophyllene. *Phlomis lychnitis* emitted α-pinene, trans-β-ocimene, and trans-β-caryophyllene. Flowers of the five species measured also emitted different amounts of diverse short-chained VOCs, such as acetic acid, ethanol, acetaldehyde, acetone and isoprene (electronic appendix).

Biscutella laevigata, *P. lychnitis*, and *E. flavicoma* had the highest visitation rates (fig. 3). *Muscari neglectum* was poorly visited by a few species of pollinators. *Rosmarinus officinalis* produced many flowers but had the lowest visitation rate per flower, visited mainly by bees and bumblebees (41 and 51%, respectively) but also by dipterans (especially Syrphidae). *Muscari neglectum* was visited mainly by bees (80%) but also by bumblebees and lepidopterans (Sphyngidae). *B. laevigata* was mainly visited by bees and coleopterans (43 and 40%, respectively) but also by ants, lepidopterans (Satyridae and Pieridae), and dipterans. *Euphorbia flavicoma* is a myrmecophilous species that was visited mainly by ants (88%) but also by dipterans (11%). *Phlomis lychnitis* was mainly visited by bees and ants (44 and 38%, respectively).

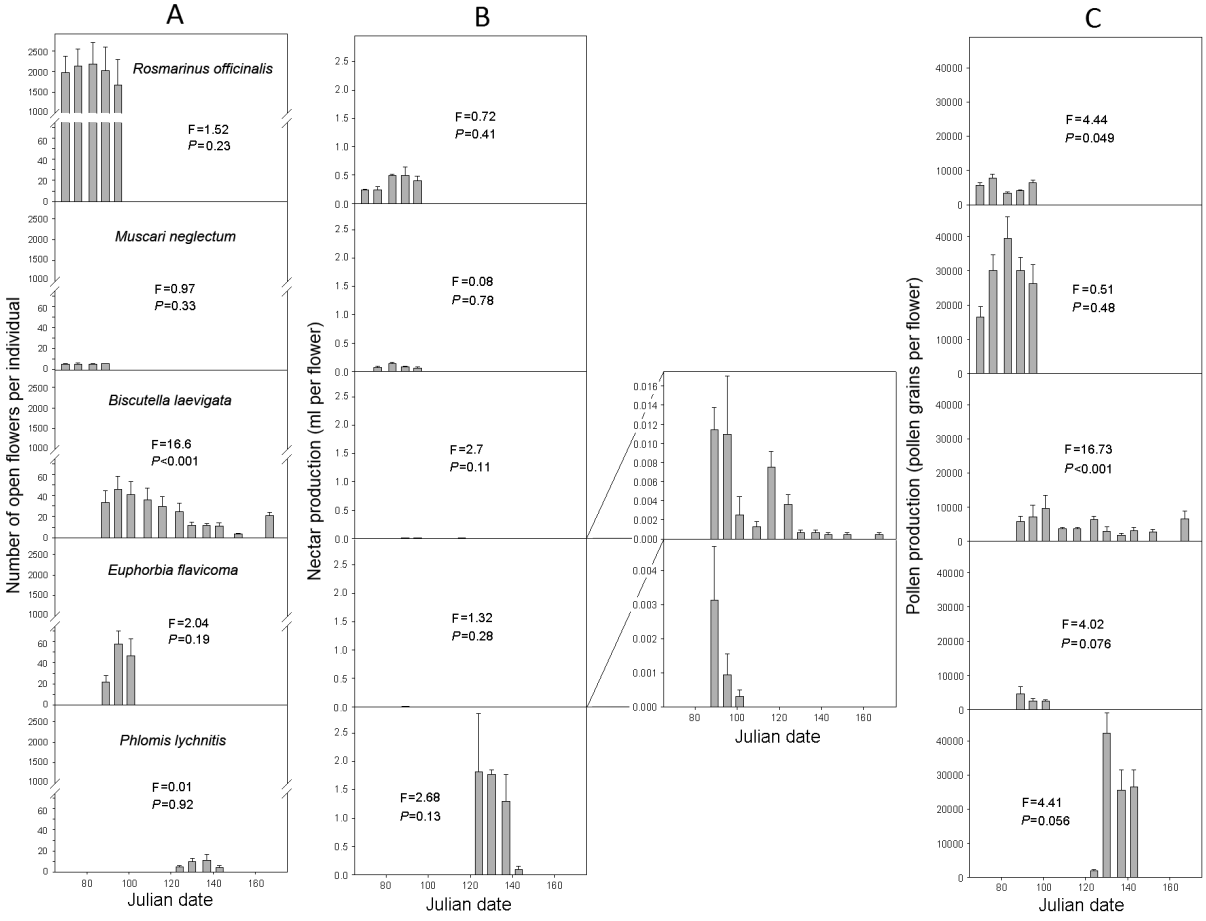

Figure 1 – A, mean values of total number of flowers per individual of the five plant species during their flowering periods in year 2011; B, mean values of nectar production per flower of the five plant species during their flowering periods in year 2011; C, mean values of pollen production per flower of the five plant species during their flowering periods in year 2011. Error bars indicate SEM (n = 5). F statistics and *P* values from the regression between flower variables and day of the year are also depicted.

Phenology of rewards, volatile emissions, and visits in the five species

Nectar production decreased significantly throughout the flowering period in *B. laevigata* (F = 16.73, $P < 0.001$) and tended to decrease in *E. flavicoma* (F = 4.02, $P = 0.076$) and *P. lychnitis* (F = 4.41, $P = 0.056$) (fig. 1B), while it increased in *R. officinalis* (F = 4.44, $P = 0.049$). Pollen production showed no defined pattern of variation in any species, although it tended to decrease (F = 2.70, $P = 0.11$) in *B. laevigata* after a maximum in mid-spring (fig. 1C). Terpene emission capacities did not gradually decrease throughout the flowering period as they did during spring at the community level (Filella et al. 2013). Terpene emission capacity tended to increase over time in *B. laevigata* (F = 2.88, $P = 0.096$) (fig. 2), coinciding with increasing temperatures (fig. 4). No significant patterns in emission rates were seen for isoprene or oxygenated short-chain BVOCs (electronic appendix). The visitation rates of pollinators increased over time in *B. laevigata* (F = 6.15, $P = 0.017$) and *P. lychnitis* (F = 11.42, $P = 0.005$) (fig. 3).

The short-period-flowering species *R. officinalis*, *M. neglectum*, *E. flavicoma*, and *P. lychnitis* did not develop any clearly consistent pattern, but the phenology of floral rewards and visitation rates in the species with a longer flowering period, *B. laevigata*, presented a pattern (fig. 4) similar to that previously observed at the community level by Filella et al. (2013; see fig. 1 in the cited paper). Number of flowers and production of pollen and nectar decreased, while visitation rates tended to increase late in the season (second half of the flowering period). Terpene emission rates in *B. laevigata* were low and did not vary throughout the flowering period, in contrast with the variation in rates among the different species observed at the community level by Filella et al. (2013).

Relation of floral rewards and volatile emissions with visitation rates

Visitation rates showed a significant negative correlation with pollen and nectar in the case of *B. laevigata* (F = 7.18, $P = 0.01$ and F = 4.64, $P = 0.037$, respectively). Floral rewards showed no significant correlations with visitation rates in the other species ($P > 0.05$). Visitation rates were found to

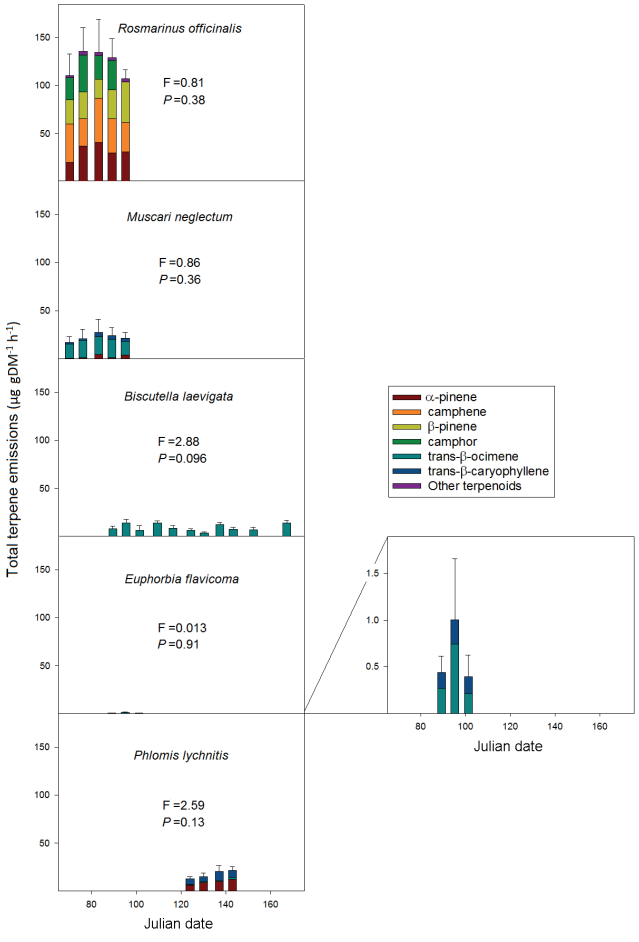

Figure 2 – Mean values of the capacity of total and individual terpene emissions of the five plant species during their flowering periods in year 2011. Error bars indicate SEM (n = 5). F statistics and *P* values from the regression between number of flowers and day of the year are also depicted.

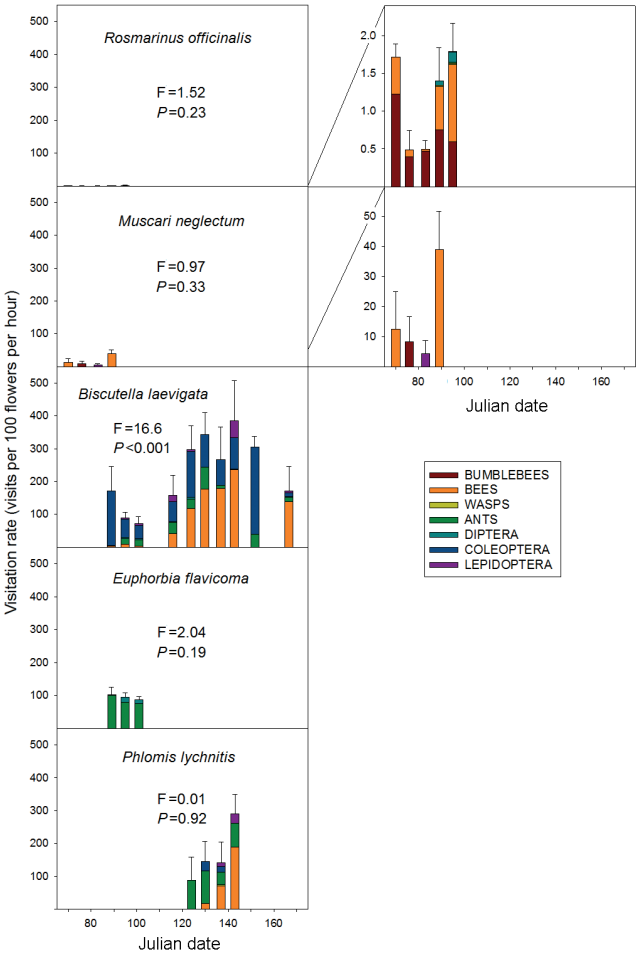

Figure 3 – Mean values of insect visitation rates for the five plant species during their flowering periods in year 2011. Error bars indicate SEM (n = 5). F statistics and *P* values from the regression between number of flowers and day of the year are also depicted.

present a significant positive correlation with terpene emission capacities in *M. neglectum* (F = 5.22, *P* = 0.03). The generalized linear mixed model conducted with pollinator visitation rates as the response variable, and with pollen and nectar production, terpene emission capacity and temperature as explanatory variables, was found significant for *B. laevigata*, and floral rewards were the variables that entered into the model (pollen: F = 8.32, *P* = 0.006; nectar: F = 3.32, *P* = 0.08, whole model: F = 2.82, *P* = 0.01). This multivariate analysis conducted for *B. laevigata* thus support the results found with univariate analyses in the same species, i.e. that nectar and pollen were the only variables that showed a significant correlation with visitation rates, and that this correlation is negative.

DISCUSSION

We observed no significant quantitative or qualitative variation in the capacities of emission of terpenes, even in *B. laevigata*. Although terpene emission rates are affected by environmental conditions, especially by temperature and humidity (Jacobsen & Olsen 1994, Llusia & Peñuelas 1999, Peñuelas & Llusia 2001, Farré-Armengol et al. 2014), the potential terpene emissions did not increase with time and therefore with increasing temperature (fig. 4). Emission capacities did not decrease with increasing pollinator abundance as would be expected for saving an appreciable amount of resources without decreasing fitness. Additionally to terpenes, we found emissions of acetic acid, ethanol and acetaldehyde, three compounds related to plant VOC catabolism (Oikawa & Lerdau 2013). These compounds are typically emitted during the fermentation of nectar by microorganisms such as yeasts reported to be present in nectaries (Herrera et al. 2009). However, the emission rates of these compounds did not correlate with nectar abundance during the flowering period of the measured species. The emission rates of short-chain oxygenated BVOCs (electronic appendix) from flowers were high compared with those from leaves (Seco et al. 2007).

We observed a similar temporal pattern between nectar and pollen production per flower in the long-period flowering species *B. laevigata* and nectar and pollen abundance at the community level as that measured by Filella et al. (2013) (fig. 4). These results support our hypothesis that decreasing competitive pressure for the attention of pollinators drives a decrease in floral investment in rewards. The short-period flowering species, *R. officinalis*, *M. neglectum*, *E. flavicoma*, and *P. lychnitis*, did not develop patterns except for a trend to decrease nectar production in species that flowered in late spring and early summer. We observed a definite trend of decreasing production of rewards especially in the species with the longest flowering period, *B. laevigata*, which was the only species that completely included the main change in the plant-pollinator market within its flowering period.

A significant negative correlation was found between floral rewards (nectar and pollen) and visitation rates in the case of *B. laevigata*. The pattern that we hypothesized for floral rewards was thus accomplished in this long-flowering

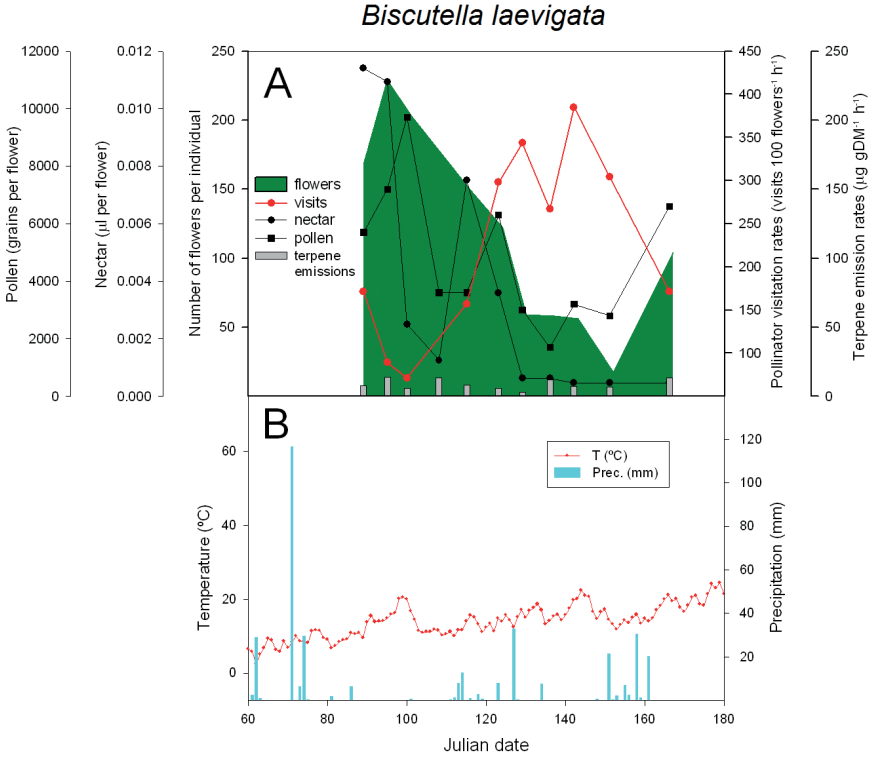

Figure 4 – A, phenology of the number of flowers, nectar and pollen production, terpene emission rates, and pollinator visitation rates for *B. laevigata* in year 2011. Values are means of the five individuals sampled each week; B, mean temperature of the day (°C) and accumulated precipitation of the day (mm) at the study area for the studied period.

species. These variables were differently affected by climatic conditions. The abundance of insects and their activity were positively correlated with temperature, but temperature alone could not account for the trends observed in visitation rates. These rates ultimately decreased in both intraspecific and community-level measurements (fig. 4, fig. 1 in Filella et al. 2013, respectively). The capacity of a plant to invest resources in floral rewards may also depend on temperature and precipitation (Carroll et al. 2001), which affect the moisture of the soil and evapotranspiration in the plant, two conditions that affect in large part the physiological state and carbon balance of a plant and are limiting factors in Mediterranean communities. In particular, nectar volume and concentration are affected by the relative humidity of the air (Corbet et al. 1979) and even vary daily in the same flower (Bertsch 1983). The inverse seasonal patterns found between the production of floral rewards and frequency of visits may in part be due to their opposite relationships with the warm and drought conditions of summer in Mediterranean communities, but they can also arise from evolutionary forces tending to increase the rewards when the pollinator visitation rates are low and there are many other coexisting plants in flower offering similar rewards to compete for pollinator attention (beginning of spring) (Cohen & Shmida 1993). Pollen production may besides be affected by other selective pressures related to the basic function of pollen as dispersive particles of fertilisation.

The differences that exist among the spectra of flower visitors of some of the species studied here may imply differences in the degree of competition for pollinator attention that these species experience. For example, *E. flavicoma* which is a myrmecophilous plant species, basically pollinated by ants, is not expected to compete with other plant species that are mainly visited by flying insects, such as *R. officinalis* or *M. neglectum*. On the other hand, *R. officinalis*, *M. neglectum*, *B. laevigata* and *P. lychnitis* all share several bee species as a significant fraction of their floral visitors, thus revealing the potential existence of a strong competition among them for the attraction of their shared pollinators, especially when their flowering periods overlap and the abundance of these pollinators is scarce (Filella et al. 2013). Furthermore, competition is not the only phenomenon that can affect the distribution of pollinator visits among coexisting plant species in a community. Facilitation, for example, can exert the opposite effect to competition, thus making high flower densities beneficial for coflowering species. The complexity of factors driving pollinator visit distribution among plant species in a flowering community adds difficulty to discern the exact role of competition.

In summary, while floral VOC emission capacities did not show a pattern of decrease in any species, the production of floral rewards generally decreased throughout the flowering period in *B. laevigata*, while the visitation rates of pollinators increased in this species. These results would support the hypothesis of a decreasing competitive pressure for the attention of pollinators that may drive a decrease in floral investment in insect rewards but not the hypothesis of a possible accompanying decrease of the capacity of emitting floral VOC olfactory signals. We detected these patterns in rewards and visits of pollinators especially in the species with the longest flowering period, *B. laevigata*, which experienced the main changes in the plant-pollinator market. Nectar production decreased in *B. laevigata* and *E. flavicoma*. The visitation rates of pollinators increased in *B. laevigata* and *P. lychnitis*. Insect abundance and activity increased with temperature throughout spring and summer, but the capacity of plants to invest resources in nectar may also be negatively affected by drier conditions occurring in late spring and summer. The negative correlation between production of floral rewards and visitation rates may thus be also induced by climatic conditions making it difficult to discern possible evolutionary forces tending to decrease rewards when pollinator visitation rates are high.

Our study provides abundant information on the floral traits of five common Mediterranean species belonging to diverse plant families and characterises the spectra of their floral visitors. This study further provides a phenological perspective for all these variables. Such information should be useful for future research, especially the data for floral VOC emissions, which enhances our knowledge of the composition of floral emissions of typical plants present in Mediterranean communities.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of mean values of acetic acid, ethanol, acetaldehyde, acetone and isoprene emissions of the five plant species during their flowering periods obtained by dynamic headspace measurements conducted with PTR-MS.

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REFERENCES

Baldwin I.T., Preston C., Euler M., Gorham D. (1997) Patterns and consequences of benzyl acetone floral emissions from Nicotiana attenuata plants. Journal of Chemical Ecology 23: 2327–2343. http://dx.doi.org/10.1023/B:JOEC.0000006677.56380.cd

Bertsch A. (1983) Nectar production of Epilobium angustifolium L. at different air humidities; nectar sugar in individual flowers and the optimal foraging theory. Oecologia 59: 40–48. http://dx.doi.org/10.1007/BF00388069

Bosch J., Retana J., Cerdà X. (1997) Flowering phenology, floral traits and pollinator composition in a herbaceous Mediterranean plant community. Oecologia 109: 583–591. http://dx.doi.org/10.1007/s004420050120

Bosch J., Martín A.M., Rodrigo A., Navarro D. (2009) Plant-pollinator networks: adding the pollinator's perspective. Ecology Letters 12: 409–419. http://dx.doi.org/10.1111/j.1461-0248.2009.01296.x

Carroll A.B., Pallardy S.G., Gallen C. (2001) Drought stress, plant water status, and floral trait expression in fireweed, Epilobium angustifolium (Onagraceae). American Journal of Botany 88: 438–446. http://dx.doi.org/10.2307/2657108

Chittka L., Raine N.E. (2006) Recognition of flowers by pollinators. Current Opinion in Plant Biology 9: 428–435. http://dx.doi.org/10.1016/j.pbi.2006.05.002

Chittka L., Thomson J.D., Waser N.M. (1999) Flower constancy, insect psychology, and plant evolution. Naturwissenschaften 86: 361–377. http://dx.doi.org/10.1007/s001140050636

Cohen D., Shmida A. (1993) The evolution of flower display and reward. Evolutionary Biology 27: 197–243. http://dx.doi.org/10.1007/978-1-4615-2878-4_6

Corbet S.A., Unwin D.M., Prys-Jones O.E. (1979) Humidity, nectar and insect visits to flowers, with special reference to Crataegus, Tilia and Echium. Ecological Entomology 4: 9–22. http://dx.doi.org/10.1111/j.1365-2311.1979.tb00557.x

Di Giusto B., Bessière J., Guéroult M., Lim L.B.L., Marshall D.J., Hossaert-McKey M., Gaume L. (2010) Flower-scent mimicry masks a deadly trap in the carnivorous plant Nepenthes rafflesiana. Journal of Ecology 98: 845–856. http://dx.doi.org/10.1111/j.1365-2745.2010.01665.x

Dötterl S., Jürgens A. (2005) Spatial fragrance patterns in flowers of Silene latifolia: Lilac compounds as olfactory nectar guides? Plant Systematics and Evolution 255: 99–109. http://dx.doi.org/10.1007/s00606-005-0344-2

Dötterl S., Jahreiß K., Jhumur U.S., Jürgens A. (2012) Temporal variation of flower scent in Silene otites (Caryophyllaceae): a species with a mixed pollination system. Botanical Journal of the Linnean Sociecty 169: 447–460. http://dx.doi.org/10.1111/j.1095-8339.2012.01239.x

Dudareva N., Negre F., Nagegowda D.A., Orlova I. (2006) Plant volatiles: recent advances and future perspectives. Critical Reviews in Plant Sciences 25: 417–440. http://dx.doi.org/10.1080/07352680600899973

Eltz T., Whitten W.M., Roubik D.W., Lisenmair K.E. (1999) Fragrance collection, storage, and accumulation by individual male orchid bees. Journal of Chemical Ecology 25: 157–176. http://dx.doi.org/10.1023/A:1020897302355

Elzinga J.A., Atlan A., Biere A., Giglord L., Weis A.E., Bernasconi G. (2007) Time after time: flowering phenology and biotic interactions. Trends in Ecology & Evolution 22: 432–439. http://dx.doi.org/10.1016/j.tree.2007.05.006

Farré-Armengol G., Filella I., Llusia J., Peñuelas J. (2013) Floral volatile organic compounds: between attraction and deterrence of visitors under global change. Perspectives in Plant Ecology, Evolution and Systematics 15: 56–67. http://dx.doi.org/10.1016/j.ppees.2012.12.002

Farré-Armengol G., Filella I., Llusia J., Niinemets Ü., Peñuelas J. (2014) [Early View] Changes in floral bouquets from compound-specific responses to increasing temperatures. Global Change Biology. http://dx.doi.org/10.1111/gcb.12628

Filella I., Bosch J., Llusia J., Peñuelas A., Peñuelas J. (2011) Chemical cues involved in the attraction of the oligolectic bee Hoplitis adunca to its host plant Echium vulgare. Biochemical Systematics and Ecology 39: 498–508. http://dx.doi.org/10.1016/j.bse.2011.07.008

Filella I., Primante C., Llusia J., Martín González A.M., Seco R., Farré-Armengol G., Rodrigo A., Bosch J., Peñuelas J. (2013) Floral advertisement scent in a changing plant-pollinators market. Scientific Reports 3: 3434. http://dx.doi.org/10.1038/srep03434

Flamini G., Tebano M., Cioni P.L. (2007) Volatiles emission patterns of different plant organs and pollen of Citrus limon. Analytica Chimica Acta 589: 120–124. http://dx.doi.org/10.1016/j.aca.2007.02.053

Gershenzon J. (1994) Metabolic costs of terpenoid accumulation in higher plants. Journal of Chemical Ecology 20: 1281–1328. http://dx.doi.org/10.1007/BF02059810

Ghazoul J. (2006) Floral diversity and the facilitation of pollination. Journal of Ecology 94: 295–304. http://dx.doi.org/10.1111/j.1365-2745.2006.01098.x

Heinrich B., Raven P.H. (1972) Energetics and pollination ecology. Science 176: 597–602. http://dx.doi.org/10.1126/science.176.4035.597

Herrera C.M., de Vega C., Canto A., Pozo M.I. (2009) Yeasts in floral nectar: a quantitative survey. Annals of Botany 103: 1415–1423. http://dx.doi.org/10.1093/aob/mcp026

Irwin R.E., Adler L.S., Brody A.K. (2004) The dual role of floral traits: pollinator attraction and plant defense. Ecology 85: 1503–1511. http://dx.doi.org/10.1890/03-0390

Jacobsen H.B., Olsen C.E. (1994) Influence of climatic factors on emission of flower volatiles in situ. Planta 192: 365–371. http://dx.doi.org/10.1007/BF00198572

Johnson S.D., Steiner K.E. (2000) Generalization versus specialization in plant pollination systems. Trends in Ecology & Evolution 15: 140–143. http://dx.doi.org/10.1016/S0169-5347(99)01811-X

Johnson S.D., Hobbhahn N. (2010) Generalized pollination, floral scent chemistry, and a possible case of hybridization in the African orchid Disa fragrans. South African Journal of Botany 76: 739–748. http://dx.doi.org/10.1016/j.sajb.2010.07.008

Kegge W., Pierik R. (2010) Biogenic volatile organic compounds and plant competition. Trends in Plant Science 15: 126–132. http://dx.doi.org/10.1016/j.tplants.2009.11.007

Kessler A., Halitschke R. (2009) Testing the potential for conflicting selection on floral chemical traits by pollinators and herbivores: predictions and case study. Functional Ecology 23: 901–912. http://dx.doi.org/10.1111/j.1365-2435.2009.01639.x

Knudsen J.T., Eriksson R., Gershenzon J., Ståhl B. (2006) Diversity and distribution of floral scent. The Botanical Review 72: 1–120.

Li P., Luo Y., Bernhardt P., Kou Y., Perner H. (2008) Pollination of Cypripedium plectrochilum (Orchidaceae) by Lasioglossum spp. (Halictidae): the roles of generalist attractants versus restrictive floral architecture. Plant Biology 10: 220–230. http://dx.doi.org/10.1111/j.1438-8677.2007.00020.x

Lindinger W., Hansel A., Jordan A. (1998) On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS). Medical applications, food control and environmental research. International Journal of Mass Spectrometry and Ion Processes 173: 191–241. http://dx.doi.org/10.1016/S0168-1176(97)00281-4

Llusia J., Peñuelas J. (1999) Pinus halepensis and Quercus ilex terpene emission as affected by temperature and humidity. Biologia Plantarum 42: 317–320. http://dx.doi.org/10.1023/A:1002185324152

Majetic C.A., Raguso R.A., Ashman T. (2009) The sweet smell of success: floral scent affects pollinator attraction and seed fitness in Hesperis matronalis. Functional Ecology 23: 480–487. http://dx.doi.org/10.1111/j.1365-2435.2008.01517.x

Mena A., Egea Gonzales F.J., Guerra Sanz J.M., Martínez Vidal J.L. (2005) Analysis of biogenic volatile organic compounds in Zucchini flowers: identification of scent sources. Journal of Chemical Ecology 31: 2309–2322. http://dx.doi.org/10.1007/s10886-005-7103-2

Niinemets Ü. (2009) Mild versus severe stress and BVOCs: thresholds, priming and consequences. Trends in Plant Science 15: 145–153. http://dx.doi.org/10.1016/j.tplants.2009.11.008

Oikawa P.Y., Lerdau M.T. (2013) Catabolism of volatile organic compounds influences plant survival. Trends in Plant Science 18: 695–703. http://dx.doi.org/10.1016/j.tplants.2013.08.011

Peñuelas J., Llusia J. (2001) The complexity of factors driving volatile organic compound emissions by plants. Biologia Plantarum 44: 481–487. http://dx.doi.org/10.1023/A:1013797129428

Peñuelas J., Llusia J. (2002) Linking photorespiration, monoterpenes and thermotolerance in Quercus. New Phytologist 155: 227–237. http://dx.doi.org/10.1046/j.1469-8137.2002.00457.x

Peñuelas J., Llusia J. (2003) BVOCs: plant defense against climate warming? Trends in Plant Science 8: 1360–1385. http://dx.doi.org/10.1016/S1360-1385(03)00008-6

Peñuelas J., Munné-Bosch S. (2005) Isoprenoids: an evolutionary pool for photoprotection. Trends in Plant Science 10: 166–169. http://dx.doi.org/10.1016/j.tplants.2005.02.005

Peñuelas J., Staudt M. (2010) BVOCs and global change. Trends in Plant Science 15: 133–144. http://dx.doi.org/10.1016/j.tplants.2009.12.005

Peñuelas J., Filella I., Stefanescu C., Llusia J. (2005a) Caterpillars of Euphydryas aurinia (Lepidoptera: Nymphalidae) feeding on Succisa pratensis leaves induce large foliar emissions of methanol. New Phytologist 167: 851–857. http://dx.doi.org/10.1111/j.1469-8137.2005.01459.x

Peñuelas J., Llusia J., Asensio D., Munné-Bosch S. (2005b) Linking isoprene with plant thermotolerance, antioxidants and monoterpene emissions. Plant, Cell & Environment 28: 278–286. http://dx.doi.org/10.1111/j.1365-3040.2004.01250.x

Peñuelas J., Ribas-Carbó M., Giles L. (1996) Effects of allelochemicals on plant respiration and oxygen isotope fractionation by the alternative oxidase. Journal of Chemical Ecology 22: 801–805. http://dx.doi.org/10.1007/BF02033587

Petanidou T., Ellis W.N., Margalis N.S., Vokou D. (1995) Constraints on flowering phenology in a phryganic (East Mediterranean shrub) community. American Journal of Botany 82: 607–620. http://dx.doi.org/10.2307/2445419

Pichersky E., Gershenzon J. (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Current Opinion in Plant Biology 5: 237–243. http://dx.doi.org/10.1016/S1369-5266(02)00251-0

Piesik D., Łyszczarz A., Tabaka P., Lamparsky R., Bocianowsky J., Delaney K.J. (2010) Volatile induction of three cereals: influence of mechanical injury and insect herbivory on injured plants and neighbouring uninjured plants. Annals of Applied Biology 157: 425–434. http://dx.doi.org/10.1111/j.1744-7348.2010.00432.x

Pleasants J.M. (1981) Bumblebee response to variation in nectar availability. Ecology 62: 1648–1661. http://dx.doi.org/10.2307/1941519

Roulston T.H., Cane J.H. (2000) Pollen nutritional content and digestibility for animals. Plant Systematics and Evolution 222: 187–209. http://dx.doi.org/10.1007/BF00984102

Schiestl F.P., Peakall R., Mant J.G., Ibarra F., Schulz C., Franke S., Franke W. (2003) The chemistry of sexual deception in an orchid-wasp pollination system. Science 302: 437–438. http://dx.doi.org/10.1126/science.1087835

Seco R., Peñuelas J., Filella I. (2007) Short-chain oxygenated VOCs: Emission and uptake by plants and atmospheric sources, sinks, and concentrations. Atmospheric Environment 41: 2477–2499. http://dx.doi.org/10.1016/j.atmosenv.2006.11.029

Seco R., Filella I., Llusia J., Peñuelas J. (2011) Methanol as a signal triggering isoprenoid emissions and photosynthetic performance in Quercus ilex. Acta Physiologiae Plantarum 33: 2413–2422. http://dx.doi.org/10.1007/s11738-011-0782-0

Sharkey T.D., Singsaas E.L. (1995) Why plants emit isoprene. Nature 374: 769. http://dx.doi.org/10.1038/374769a0

Shmida A., Dafni A. (1989) Blooming strategies, flower size and advertising in the "Lily-group" geophytes in Israel. Herbertia 45: 111–122.

Shuttleworth A., Johnson S.D. (2009). The importance of scent and nectar filters in a specialized wasp-pollination system. Functional Ecology, 23: 931–940. http://dx.doi.org/10.1111/j.1365-2435.2009.01573.x

Vázquez D.P., Blüthgen N., Cagnolo L., Chacoff N.P. (2009) Uniting pattern and process in plant–animal mutualistic networks: a review. Annals of Botany 103: 1445–1457. http://dx.doi.org/10.1093/aob/mcp057

Vogel S. (1983) Ecophysiology of zoophilic pollination. In: Lange O.L., Nobel P.S., Osmond C.B., Ziegler H. (eds) Physiological plant ecology III: 559–624. Berlin, Springer-Verlag. http://dx.doi.org/10.1007/978-3-642-68153-0_16

Wright G.A., Schiestl F.P. (2009) The evolution of floral scent: the influence of olfactory learning by insect pollinators on the honest signalling of floral rewards. Functional Ecology 23: 841–851. http://dx.doi.org/10.1111/j.1365-2435.2009.01627.x

Wyatt R., Broyles S.B., Derda G.S. (1992) Environmental influences on nectar production in milkweeds (Asclepias syriaca and A. exaltata). American Journal of Botany 79: 636–642. http://dx.doi.org/10.2307/2444879

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