Universitat Autònoma de Barcelona

The role of floral traits in structuring plant-pollinator interactions

Tesi doctoral

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Per optar al grau de Doctora

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Contents

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Resum

Les interaccions planta-pol·linitzador són un component essencial de la biodiversitat i la funció ecològica dels ecosistemes terrestres. Un dels principals objectius de l'ecologia de la pol·linització és descriure aquestes interaccions i comprendre els factors subjacents a la seva estructura. En aquesta tesi doctoral es presenten els resultats dels estudis duts a terme durant tres anys en una comunitat mediterrània de plantes i els seus insectes pol·linitzadors al parc natural del Garraf (NE Espanya). En el capítol 1 es van registrar les interaccions planta-pol·linitzador i es van mesurar diferents trets morfològics, fenològics i ecològics de les especies de plantes i pol·linitzadors en un intent d'establir quins d'aquest trets estructuren les relacions planta-pol·linitzador. Es van registrar 14.713 contactes entre les principals 23 espècies de plantes i 221 espècies de pol·linitzadors, que van representar 960 interaccions específiques. Trobem que un tret ecològic (densitat de flors) i un tret fenològic (temps de floració) de les plantes van ser els principals factors que expliquen les interaccions observades. Trets florals, com la restricció de la corol·la i el pol·len i la producció de nèctar per flor, van tenir un efecte menor. En el capítol 2 s'explora el paper d'un atribut floral diferent i complex, l'aroma floral, en l'estructuració de la variació temporal de les taxes de visites de pol·linitzadors en la mateixa comunitat. Trobem que les plantes que floreixen a principis de la temporada, en un moment en què les flors són més abundants però els pol·linitzadors són escassos, produeixen major quantitats de compostos volàtils que les plantes que floreixen més tard, quan els pol·linitzadors són molt més abundants en relació a les poques flors disponibles. Aquest és el primer estudi en el qual s'analitzen els patrons d'emissió de fragàncies florals a nivell comunitari. Finalment, al capítol 3 s'exploren amb més detall la importància d'aquestes fragàncies florals mitjançant un cas d'estudi realitzat en el Jardí Botànic i Ecològic de la Universitat de Bayreuth (Alemanya). En aquest estudi es va utilitzar una planta focal (la composta *Cirsium arvense*) i un dels seus pol·linitzadors principal (el sírfid *Episyrphus balteatus*). Mitjançant mesures de volàtils al laboratori, tècniques d'electroantenografia i bioassajos es

va trobar que els senyals olfactius eren més importants que els senyals visuals com a atraients de *E. balteatus* cap a les inflorescències de *C. arvense*.

Abstract

Plant-pollinator interactions are an essential component of biodiversity and ecological function in terrestrial ecosystems. One of the main objectives of pollination ecology is to describe these interactions and to understand the factors underlying their structure. In this PhD thesis we present the results of studies conducted over three years in a Mediterranean plant-pollinator community in the natural park of el Garraf (NE Spain). In chapter 1 we monitored plant-pollinator interactions and measured plant and pollinator traits in an attempt to establish the main drivers of plant-pollinator relationships. We recorded 14713 contacts between the main 23 plant species and 221 pollinator species, representing 960 specific interactions. We found that an ecological trait (flower density) and a phonological trait (flowering time) were the main factors explaining the observed interactions. Floral traits, such as corolla restrictiveness and pollen and nectar production per flower, had a lesser effect. In chapter 2 we explore the role of a different and complex floral attribute, floral scent, in structuring temporal variation in pollinator visitation rates in the same community. We found that plants blooming early in the season, at a time when flowers are most abundant but pollinators are scarce, produce larger amounts of volatiles than plants blooming later, when pollinators are plentiful for the few flowers available. This is the first study in which emission patterns are analysed at the community level. Floral fragrances are further explored in chapter 3, which describes a case study conducted at the Ecological Botanical Garden of the University of Bayreuth (Germany). This study involves a focal plant (the composite *Cirsium arvense*) and a focal pollinator (the syrphid fly *Episyrphus balteatus*), and uses laboratory volatile measurements, electroantennography techniques and biossays. We found that olfactory cues were more important than visual cues as attractants of *E. balteatus* to *C. arvense* inflorescences.

General Introduction

Interactions between plants and animals are of a capital importance for ecosystem function since they channel the flow of nutrients and energy between producers and consumers. Plants interact with a vast spectrum of animals ranging from simple microorganisms to complex vertebrates. Interactions may be positive (mutualism, symbiosis) or negative (antagonistic interactions) (Begon, Townsend & Harper 2005). This polarity depends on the benefits the two actors obtain from the interaction. Herbivory and florivory for example represent negative interactions for the plant, but positive for the animal; pollination and seed-dispersion, on the other hand, are usually positive interactions for both actors (C. M. Herrera & Pellmyr 2002). The limited mobility of plants is the key for understanding the plants' need for animal vectors in mutualistic interactions. Among mutualistic interactions, pollination is of central importance since it results in seed and fruit production, thus setting the stage for subsequent mutualistic (seed dispersal) and antagonistic interactions (seed predation, herbivory) involving new actors. Plant-pollinator interactions are therefore key contributors to biodiversity and essential to ecosystem maintenance (Kearns, Inouye & Waser 1998). The recently documented pollinator declines due to global change or thus of great concern, as they may have severe repercussions on plant fertility, fruit and seed set and population dynamics (Louda 1982; Rathcke & Jules 1993; Aizen & Feinsinger 1994; Kearns & Inouye 1997; Ashmann et al. 2004). Ultimately, these declines may compromise ecosystem services (Ollerton et al. 2011; Vanbergen et al. 2013).

Plants have evolved specific structures to interact with animal vectors: flowers. By means of flowers the pollination interaction ensures plant reproduction and food intake by animal vectors. The astonishing diversity of flowers in angiosperms has always fascinated the human eye. One of the main goals in pollination biology is to understand the origin and the function of the enormous trait variability between flowers. Much of this diversity has been attributed to

adaptation to different pollinators; what has been called the *adaptive radiation* of angiosperms. As such, pollination interactions are considered a mechanism of speciation in plants and pollinators (Proctor et al. 1996; Kearns & Inouye 1997; Johnson & Steiner 2000; Steffan-Dewenter et al. 2005). Flowers emit signals to pollinators that are mediated by floral traits (Raguso 2004; Schaefer et al. 2004). By means of its pollinating effectiveness, a pollinator may act as a selective agent on floral traits (Gómez et al. 2008 and references therein). Classically, some floral traits (colour, corolla morphology, rewards production) have received more attention as drivers of plant-pollinator interactions (Sakai et al. 1999; Hingston & McQuillan 2000; Wilson et al 2004; Wolfe & Sowell 2006; Smith et al. 2008; Marten-Rodriguez et al. 2009). Other traits (flower scent, phenology, abundance), on the other hand, have received much less attention. Flower scent is one of the main traits considered in this thesis. Fragrance is a complex component of the floral phenotype involved primarily in communication between flowering plants and their pollinators. It promotes specialization in plant-pollinator relationships (via private channels of unusual compounds), as well as out-crossing and reproductive isolation (via flower constancy) (Raguso 2008). As any other floral trait, flower scent may be under selective pressure, but perhaps due its intrinsic complexity, scent and olfaction are rarely integrated in studies on the ecology and evolution of plant-pollinator interactions. With one exception (proboscis length) (Brian 1957; Heinrich 1979; Lack 1982; Pleasants 1983; Prys-Jones & Corbet 1987; Inoue & Kato 1992; Fussell & Corbet 1992), pollinator traits have been less often considered. Surprisingly, following pioneering studies on pollination energetics (Heinrich 1975), the potential role of pollinator body size as a potential driver of plant-pollinator interactions remains largely unexplored. Body size is therefore, another trait considered in this thesis.

The context in which interactions are studied is of upmost importance. The classical view is to consider one or few plants and their pollinators. However, plants species share pollinators with other co-occurring, co-flowering plant species, with which they may establish indirect interactions (competition and

facilitation for pollinators) (Rathcke 1983). Although the emphasis has traditionally been on tightly co-evolved plant-pollinator interactions (Proctor et al. 1996), most plants are visited by wide arrays of unrelated pollinators, which, in turn, are usually highly opportunistic and visit several plant species within a community (Waser et al. 1996). As a consequence, coevolution between plants and animals must be understood as a diffuse process, rather than a pairwise process (J. Herrera 1988). For this reasons, a community approach is necessary to fully understand the factors underlying plant-pollinator interactions. The few studies that have analyzed the role of biological and ecological traits in structuring plant-pollinator interactions have found a weak structure and some significant traits, not necessarily coincidental from study to study (McCall & Primack 1992; Hingston & McQuillan 2000; J. Herrera 1988; Bosch et al. 1997; Dicks et al. 2002; Hegland & Totland 2005). More recently, the network approach has provided much promising new insights into the structure of plant-pollinator relationships (Bascompte et al. 2003; 2006; Jordano et al. 2003; Vázquez & Aizen 2003; 2004; Blüthgen et al. 2007; Alarcón et al. 2008; Bosch et al. 2009; Vázquez et al. 2009), although the integration of biological attributes into pollination network structure is still in its infancy (Stang et al. 2006; 2009; Junker et al. 2012; Bartomeus 2013).

In this thesis we study plant-pollinator relationships in a Mediterranean community in the natural park of El Garraf, (NE Spain). The overall objective of the thesis is to understand the relative importance of various plant and pollinator attributes in explaining the structure of plant-pollinator interactions. Mediterranean ecosystems host a large species diversity (especially of plants and insects) in proportion to the small area they occupy (Mittermeier et al. 1999, Blondel & Aronson 1999). Mediterranean ecosystems are good models for plant-pollinator interaction studies because both flowering periods and pollinator activity are highly seasonal (Bosch et al. 2009). In addition, flowering periods and pollinator activity periods are strongly affected by abiotic factors (temperature, rainfall, sunlight) that fluctuate across time and seasons (J. Herrera 1986, Arroyo

1990), which may prevent the evolution of tight plant-pollinator interactions. In addition, many disturbance factors (wild fires, fragmentation, land-use change, biological invasions) are strongly affecting Mediterranean communities (Potts et al. 2003; Bartomeus et al 2008). A better understanding of plant-pollinator interactions in Mediterranean communities is therefore important to guide land use management and conservation efforts in the Mediterranean basin.

The thesis is structured as follows:

In **Chapter 1** we consider the role of floral traits and pollinator traits in organizing a Mediterranean plant-pollinator community. With this purpose, plant-pollinator interactions were monitored during three years in a plant pollinator-community composed of 23 plant and 221 pollinator species. To understand the factors responsible for the observed plant-pollinator interaction structure, several flower traits (flower density, floral display, corolla depth, pollen production, nectar production, flowering phenology), along with pollinator traits (proboscis length, body size, activity phenology) were measured. Most studies of this kind address the relationship between flower traits and pollinator assemblage composition. The approach we follow attempts to establish the relationship between flower traits and pollinator traits.

In **Chapter** 2 we study the dynamics of flower scent throughout the flowering period of the community. We follow a biological market approach, in which plants use scent to advertise their floral resources to potential buyers (pollinators). With this purpose we analyzed the seasonality of emissions of the entire community and related it to pollinator visitation rates. Special emphasis was place on a specific class of floral volatiles, terpenes, which are common compounds of flowers pollinated by bees. This study represents the first attempt to characterize flower scent emissions at the community level.

In **Chapter 3** the role of floral scent in pollinator attractiveness is explored with greater depth. This chapter represents a case study on a focus plant (*Cirsium arvense*) and a focal pollinator, the hoverfly (*Episyrphus balteatus*), and was conducted during two stays at the University of Bayreuth. We explored the relative importance of visual and olfactory cues of *C. arvense* as attractants of *E. balteatus* syrphid. It is generally assumed that hoverflies use visual cues for flower location, but little is known about the contribution of floral scent. In this chapter we present a set of bioassays and laboratory measurements on *C. arvense* flower volatile emissions and *E. balteatus* antennal detection. We reveal, for the first time, the importance of olfactory over visual cues in non-yellow flowers.

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Chapter 1

Flower and pollinator traits organize pollination interactions in a Mediterranean community

Primante C., Rodrigo A., Barril-Graells H., Martín González A. M., Bosch J. (in preparation).

Flower and pollinator traits organize pollination interactions in a Mediterranean community

Abstract

One of the long term objectives of pollination ecology is to understand how pollinators partition their visits among the flowers available to them. Traditionally, attention has focused on flower traits, but pollinator traits such as feeding requirements, body size and proboscis length are also important in structuring plant-pollinator interactions. Pollination syndrome theory has provided a simplified ordination of plant-pollinator relationships and a mechanistic explanation for the evolution of flower traits, but has failed to explain the pervasiveness of generalist interactions and their lability across time and space. In this study we monitor plant-pollinator interactions in a Mediterranean scrubland and measure plant and pollinator traits in an attempt to establish the main drivers of plant-pollinator relationships. We recorded 14713 contacts between the 23 plant species and 221 pollinator species, representing 960 specific interactions. Ordination analyses relating floral traits and pollinator functional groups explained 31% of the observed variance. Most of this variance was explained by flower density and blooming time. A lower proportion of the variance was explained by corolla restrictiveness and pollen nectar/nectar production. Cluster analysis yielded three pollinator groups. The first cluster is dominated by female bees and Diptera, and is associated with plants with high flower density and early blooming period. The second cluster is dominated by Coleoptera and female bees, and is weakly associated with flowers producing large amounts of pollen and, to a lesser extent, nectar. The third cluster is mostly composed of nectarivorous Hymenoptera (male bees and wasps) and is weakly linked to flowers with restrictive corollas. We found a weak relationship between proboscis length and corolla restrictiveness, but body size and pollen/nectar production per flower were not correlated. Our results do not support pollination syndrome theory. Most

plant species in our community are wide generalists and pollinator attraction seems to be more dependent on pollinator traits than on taxonomic status. Differences among plants in flower traits may be too small in Mediterranean communities to elicit large differences in pollinator spectra. Any effect of floral traits may be overridden by ecological traits and seasonal patterns, thus hindering the appearance of well-defined pollination syndromes.

1.1 Introduction

Plant-pollinator interactions usually result in a mutual benefit for both actors. Pollinators satisfy their energetic requirements to sustain day-to-day life and reproduce, while plants experience pollination through pollen receipt and pollen export. The effectiveness of this mutualistic process depends on suites of both plant and pollinator traits, including morphological, phenological and behavioural attributes. Plant-pollinator communities are typically composed of tens of plant species and hundreds of pollinator species. One of the long term objectives of pollination ecology is to understand how this high diversity of pollinators partition their visits among the flowering plants available to them. Traditionally, attention has focused on flower traits and their overwhelming diversity, assumed to be the result of adaptation to different pollinators (Proctor et al. 1996). However, pollinator communities also display a wide diversity of morphological (body size, mouthpart length), as well as behavioural traits (feeding habits, foraging behaviour).

The first attempts to clarify plant-pollinator interactions brought about the establishment of pollination syndromes (Faegri & van der Pijl 1979, Kevan & Baker 1983). Pollination syndromes can be defined as suites of floral traits assumed to have evolved as adaptations to a specific group of pollinators, usually expressed at the level of insect orders (e.g., beetles vs. butterflies vs. bees) or above (e.g., insects vs. birds) (Waser et al. 1996, Fenster et al. 2004). Flower shape, colour and scent, but also the quantity and quality of floral rewards

and their accessibility and timing of anthesis are the classical floral traits used to define pollination syndromes. This means that, irrespective of their phylogenetic relatedness, flowers with similar features tend to attract similar groups of pollinators. This concept is the basic brick upon which the “pollination syndrome” theory is built on. One of the consequences of such a co-evolutionary process would be an increase in mutual specialization, tending to maximize fitness of both the plant and the pollinator.

The pollination syndrome concept has been successful in providing a simplified ordination of the overwhelming phenotypic flower diversity and also a mechanistic explanation for the evolution of flower traits, resulting from the selective pressure exerted by pollinators (Fenster et al. 2004, Ollerton et al. 2009). For this reason, it has been used (and is still used to some extent) to predict the optimal pollinators of plant species in absence of direct observations (Ollerton 1998, Ollerton et al. 2009 and references therein). However, pollination syndrome theory has been criticized based on two lines of evidence. First, specialization on certain groups of pollinators appears to be the exception rather than the norm. In fact, most plant species are visited by a wide array of pollinators (C.M. Herrera 1996, Waser et al. 1996, Hingston & McQuillan 2000, Aigner 2001, Ollerton et al. 2007, Marten-Rodriguez et al. 2009). Second, pollination systems are more dynamic than syndromes might predict: pollinator assemblages to a given plant species are highly variable, both among sites and from year to year (C.M. Herrera 1988, Conner & Neumeier 1995, Fishbein & Venable 1996, Wilson et al. 2004, Moeller 2005, Hegland & Boeke 2006, Lázaro et al. 2009, 2010). This suggests that plants face important fluctuations in pollinator visitation and pollinator composition, thus diluting the potential selective pressure exerted by any specific pollinator and submitting plants to different selective pressures from year to year. Under this scenario, tight co-adaptive processes seem unlikely to occur. In sum, to many critics pollination syndromes provide a systematic and ordered conceptual framework, but do not quite describe real field situations.

A further criticism to the pollination syndrome concept is its inability to place pollination systems within a proper ecological context. Plants and their pollinators do not grow in isolation from other plants and other pollinators. Instead, plants share pollinators with other co-occurring, co-flowering species, with which they may establish indirect interactions such as competition for pollination or facilitation (whereby a plant benefits from visitation from pollinators primarily attracted by another species) (Rathcke 1983). To fully understand plant-pollinator relationships it is therefore of upmost importance to work within a community context. Flower abundance and flower neighbourhood (abundance and diversity of co-flowering species) have been shown to affect pollinator visitation rates and pollinator composition (Conner and Neumeier 1995, Kunin 1997, Grindeland et al. 2005, Hegland & Boecke 2006, Lázaro et al. 2009, 2013). Thus, within a community context, ecological factors may be just as important as biological attributes in determining pollinator flower choices.

Community studies exploring the association between pollinator partitioning and flower traits have been slowly accumulating during the last decades (J. Herrera 1988; McCall & Primack 1992, Bosch et al. 1997, Hingston & McQuillan 2000, Hegland & Totland 2005, Lázaro et al. 2008). In general, these studies have found moderate levels of structure in plant-pollinator interactions, and weak but significant effects of certain flower traits on pollinator partitioning. However, the results of these studies are far from consistent, and different plant attributes appear to be relevant in different communities. A biological attribute of primary importance but sometimes overlooked is flowering phenology. Flowering phenology acts a first filter, since many potential interactions do not occur simply due to lack of overlap between flowering periods of plants and activity periods of pollinators (phenological forbidden links; Jordano et al. 2003). Phenology is particularly important in Mediterranean systems, with a strong seasonal component.

Another shortcoming of pollination syndromes is their inability to incorporate pollinator traits. Classically, pollinators are subdivided into groups merely based on taxonomical criteria (Faegri & van der Pijl 1979), irrespective, thus, of differences in morphological features and foraging behaviour. For example, proboscis length has long been shown to be an important determinant of pollinator partitioning in many systems, as species with long mouthparts favour flowers with deep corollas (Inouye 1980, Ranta & Lundberg 1980, Pleasants 1983, Nilsson 1988, Manning & Goldblatt 1997, Corbet 2000, Alexandersson & Johnson 2002, Dohzono et al. 2004, Pauw et al. 2008). Most of these studies, however, involve a particular pollinator group (usually bumblebees or butterflies), with few attempts to explore an entire community (Stang et al. 2006). Another potentially important pollinator trait is body size. Body size was an essential component of the "pollinator energetics" framework developed in the 1970's and 1980's. This ecophysiological approach was based on optimal foraging theory and the balance between foraging costs and energetic gains obtained from floral resources (Heinrich & Raven 1972, Heinrich 1975, 1979, Pyke 1978). According to this view, and due to their energy expenditure associated to flight, large-sized pollinators could only forage profitably on flowers producing large amounts of nectar. Although initially based on nectar intake, this approach was later extended to pollen rewards (Rasheed & Harder 1997a, 1997b, Harder et al. 2001). A few studies have provided empirical support for the relationship between body size and flower rewards, but mostly when comparing pollinators differing widely in body size (e.g. bees *versus* birds and mammals; Brown et al. 1978, Kodric-Brown et al. 1984, Dalsgaard et al. 2009). Thus, in spite of being a feature of extreme physiological importance (Reiss 1989, Gaston & Blackburn 2000), and in contrast to its demonstrated role in food web assemblage (Woodward et al. 2005), body size remains a poorly investigated trait in pollination ecology.

In this study we monitored plant-pollinator interactions in a Mediterranean scrubland community and measured plant and pollinator traits in an attempt to

establish the main drivers of pollinator partitioning among the plants of the community. We ask the following questions: What are the main floral traits structuring plant-pollinator interactions in the community? What is the role of pollinator traits? Do pollinators with similar flower visitation patterns share common traits? Is there a relationship between body weight and reward production? And between proboscis length and corolla restrictiveness?

1.2 Materials and methods

1.2.1 Study site

We studied a garrigue-like Mediterranean shrubland community in the Parc Natural del Garraf (Barcelona, NE Spain; coordinates: 409340.35, 4569657.08), dominated by *Quercus coccifera, Pistacia lentiscus, Rosmarinus officinalis* and *Thymus vulgaris*. We delimited a ~ 1 ha plot located located 340 m above sea level and 1700 m from the coast line. Field work was conducted in 2006, 2007 and 2008 from March to June, encompassing most of the flowering period. In July-August bloom is virtually arrested in coincidence with the summer drought. All pollinators are insects.

1.2.2 Flower transects

We laid six permanent transects of 50 m x 1 m crisscrossing the study site (total 300 m²). Once a week we counted all open flowers in them. We decided to work on the 23 most abundant entomophilous plant species, accounting for 99.7 % of the flowers counted in the transects.

1.2.3 Pollinator surveys

Pollinator counts on the 23 selected plant species were conducted two-three days per week from 10:00 to 17:00 during fair weather. On each sampling day, we tagged patches of each plant species in bloom and counted all open flowers in these patches. We then spent 4 minute intervals observing the patch and noted all pollinators visiting the flowers. All flower visitors that were observed

consuming pollen and/or nectar were considered pollinators, irrespective of their "quality" (i.e. effectiveness in transferring pollen). Sampling effort amounted to 382 hours of surveys. A few specimens of most pollinator species were captured for identification in the laboratory and to obtain morphological measures (see below).

Pollinators were grouped into six functional groups, based on taxonomic affinity, feeding habits, and foraging behavior: 1) Coleoptera (COL). Beetles of various families (Dasytidae, Buprestidae, Scarabaeidae, Mordellidae, Nitidulidae, etc.) feeding on both pollen and nectar; 2) Diptera (DIP). Flies of various families (Syrphidae, Calliphoridae, Muscidae, Anthomyidae, Bombyliidae, etc.) feeding on both pollen and nectar; 3) Lepidoptera (LEP). Butterflies and a few moths feeding on nectar; 4) Bee females (BEF). Females of Apiformes collecting both pollen and nectar; 5) Nectarivorous Hymenoptera (NEC): Wasps of various families, and males of Apiformes feeding almost exclusively on nectar; 6) Ants (ANT). Nectar consumers. 7) Other (OTH) Hemiptera and Orthoptera.

1.2.4 Flower traits

Information on flower phenology was obtained from the transect data. We characterized flowering periods according to the following variables (expressed as the mean of the three years): 1) Beginning of bloom (the earliest week of a plant species bloomed); 2) Blooming peak (week of maximum blooming intensity); 3) duration of the blooming period (number of weeks during which a species was in bloom).

Information on flower density and patch size was also obtained from the transect data. Flower density was expressed as the sum of flowers counted throughout the year / m^2 (three-year mean). Patch size was the average number of open flowers per patch at the flowering peak (three-year mean).

Corolla depth can be easily measured, but does provide a good estimate of nectar accessibility, which is also dependent on the width of corolla aperture. For example, sufficiently small insects can easily reach the nectaries of flowers with deep corollas irrespective of mouthparts length. For this reason, we preferred to use a scale of “corolla restrictiveness” ranging from 1 (open and bowl-shaped flowers), to 2 (bell shaped, short tubular and small papilionaceous flowers) and 3 (long narrow tubular and large papilionaceous flowers).

To measure the pollen production, we collected 10-15 flower buds of each species and kept in vials with 70% ethanol. In the laboratory, flower buds were individually dissected under a stereomicroscope and the number of anthers was counted. Then, three anthers per flower were removed, suspended in 2 ml of 70% ethanol and sonicated in a water bath for 2-4 minutes to dislodge pollen grains. Anther tissue was then removed and 9 ml of isotonic solution were added. The number of pollen grains in the resulting suspension was estimated using an electronic particle counter (Coulter Multisizer) with 200 μm aperture. From these data we obtained the total number of pollen grains produced per flower. In addition, we measured the diameters of 15 pollen grains per plant species, and, depending on the shape of the pollen grain used the formula of a sphere or an ellipsoid to calculate pollen grain size. Pollen grain number and size data were used to calculate volume of pollen produced per flower flower (in mm^3). This variable was then multiplied by mean patch size (open flowers per patch) to obtain pollen production per patch.

To measure nectar production we enclosed flower buds of each species with nylon bags. Twenty four hours after anthesis we extracted and measured the nectar accumulated with Drummond micropipettes of 0.25, 0.50 and 1 μl. Sample sizes were 19-144 flowers per species (mean: 51 flowers). To measure nectar concentration, we used field refractometers (Eclipse, Bellingham & Stanley). From these data we calculated sugar content per flower (expressed in mg; Dafni

1992). Results were combined with mean patch size to obtain nectar production (mg of sugar) per patch size.

1.2.5 Pollinator traits

Data from pollinator surveys were supplemented with additional field observations to obtain information on pollinator activity periods. We worked with two measures of pollinator activity: 1) Beginning of the flight period (the earliest week of the year in which a species was observed); 2) Duration of the flight period (weeks during which a species was active).

Captured pollinators were taken to the laboratory and weighed (accuracy: 0.1 mg). To minimize weight loss, specimens were weighed on the day of capture. Other studies use dry weight instead of wet weight, but both variables are highly correlated ($r = 0.97$; Agosta and Janzen 2005). We weighed a total of 1803 specimens.

Captured individuals were also used to measure mouthparts length. Mouthparts were dissected, glued to paper tags, and then measured under the stereomicroscope. In Hymenoptera, the length of the proboscis was measured as the length of the fully extended prementum and glossa. In syrphids and other dipterans, we measured the extended labium and labellum. In Lepidoptera, the proboscis was carefully unrolled and then measured. In Heteroptera, we considered the length of the fully extended rostrum. We measured a total of 1598 specimens. Mouthparts length of Coleptera, ants and small parasitic wasps was assumed to be zero. Pollinator specimens and mouthparts are deposited in the CREAF collection.

1.2.6 Data analysis

Interaction matrix

Pollinator surveys allowed us to characterize the interaction strength of each plant-pollinator interaction. Weekly interaction strength was calculated as the mean number of contacts of each pollinator species recorded per flower and minute, multiplied by weekly flower abundance. Final interaction strength was obtained as the sum of weekly interaction strengths. Then, we built an interaction matrix (where rows represent pollinator species and columns plant species) with the three-year mean interaction strengths. Ants were not included in this matrix because their abundance on a given plant was strongly conditioned by proximity to their nests. Rare pollinator species (represented by with less than 4 contacts over the three years) were also excluded in an attempt to underweight spurious interactions.

Flower variable matrix

We built a flower variable matrix with rows representing plant species and columns flower variables. We initially considered the following plant variables: blooming beginning, blooming peak, blooming duration, flower density, patch size, corolla restrictiveness, nectar production per flower, nectar production per individual patch, pollen production per flower, and pollen production per patch. We then explored the association between these variables. Flower density and patch size were highly correlated ($R= 0.9$; $p= 0.0001$), and both were found to be correlated with nectar and pollen production per patch ($R= 0.6$; $p \leq 0.002$ in all four cases), which were also highly correlated ($R= 0.9$; $p = 0.0001$). We thus decided to work with flower density and nectar and pollen production per flower. Blooming beginning was highly correlated with blooming peak ($R= 0.9$; $p= 0.0001$) and with duration of the blooming period ($R= -0.7$; $p= 0.0001$). For this reason we finally considered blooming beginning. In sum, the five flower variables finally used to characterize each flower species were: flower density, blooming beginning, corolla restrictiveness, nectar production per flower, and

pollen production per flower (Table 1). Transformation did not improve normality of these flower variables. Therefore, we worked with untransformed data since we detected no anomalies in the error distribution.

Ordination analysis

To effect of flower traits on interaction composition and strength was assessed through ordination analysis. We first run a detrended correspondence analysis (DCCA) between the interaction matrix and the flower variable matrix to determine whether our data had a unimodal or a linear response (Lepš & Šmilauer 2003). The results of this analysis showed that our data were sufficiently homogeneous and conformed to a model with a linear response (gradient length = 3.4). We thus run a Redundancy Analysis (RDA) with the two matrices. We worked with centred and untransformed data. In addition, we standardized our data in order to homogenise the effect of the different pollinator species, irrespective of their interaction strength. Flower variables were automatically selected with the *forward* option, and significance of each variable as well as significance of the model was tested with Monte Carlo simulations under reduced model (499 permutations). These analyses were conducted with Canoco v.4.5 (ter Braak & Šmilauer 2002).

From the coordinates of the first four axes obtained in the RDA, pollinator species were grouped using cluster hierarchical methods (HCLUST function) (unpaired-group-method-analysis, UPGMA, based on Euclidian distances). The cluster analysis was conducted with STATS package in R (R 3.0.1 Development Core Team 2010). We wanted to test if the three resulting pollinator groups (see results) differed in biological traits. Each pollinator species was characterized according to body weight, proboscis length, and beginning of the activity period. Duration of the activity period was not considered because, with the virtual cessation of activity in July, species starting their activity late in the season could not possibly have long activity periods.

Differences among the three groups in activity period were analyzed with ANOVA. We worked with untransformed data. No anomalies in the error distribution were detected, confirming the goodness-of-fit of the method. Differences in body weight among the three groups were tested with ANOVA. As found in previous studies (Harder 1983, Agosta & Janzen 2005, Stang et al. 2006), proboscis length and body weight were positively correlated in our community ($R = 0.7$; $p = 0.0001$, correlation made including coleopterans). Because we were especially interested in the potential effects of proboscis length independently of body weight, we conducted and ANCOVA with proboscis length as dependent variable and body weight as a covariate. Pollinator weight and pollinator proboscis length were log-transformed to achieve normality.

1.3 Results

1.3.1 Plant traits

The 23 species surveyed showed a wide range of interspecific variation in floral traits (Table 1). Beginning of bloom ranged from the 1st and the 12th week. Flower density ranged between 0.17 and 659 flowers per m^2. About one third (35%) of the species had open corollas with readily accessible nectaries, 39% had moderately restrictive corollas, and 26% had narrow, restrictive corollas. Nectar production per flower ranged between 0 and 0.53 mg of sugar and pollen production per flower between 0.03 and 8.98 mm^3 (Table 1).

1.3.2 Pollinator community and pollinator traits

Throughout the three years, we recorded 14713 contacts between the 23 plant species and 221 pollinator species, representing 960 specific interactions. The Hymenoptera were the richest group with 99 species (56 bees, 34 wasps and 9 ants), followed by Diptera, with 52 species (18 hoverflies, 5 bee flies, 12 muscoid flies and 17 other). Coleoptera were represented by 38 species and Lepidoptera by 25. Heteroptera and Orthoptera were represented by 5 and 2 species, respectively. Because we did not consider ant interactions or interactions

represented by fewer than 4 contacts, the final numbers of contacts, interactions, and pollinator species used in the analyses were 10245, 735, and 105 respectively (46 Hymenoptera, 24 Diptera, 23 Coleoptera, 10 Lepidoptera, 1 Heteroptera and 1 Orthoptera species). Because male and female bees were separated based on their different feeding habits, the number of pollinators considered was 116 instead of 105.

Plant species	Acronyms	Blooming beginning (week)	Flower density per m^2	Corolla restrictiveness categories	Nectar production per flower (mg of sugar)	Pollen production per flower (pollen volume in mm^3)
*Allium sphaerocephalon**	ASP	11.5± 0.69	0.47± 0.7	2	0.08± 0.01	3.6± 0.32
*Anagallis arvensis**	AAR	5.3± 1.16	5.14± 4.8	1	-	0.07± 0.01
Biscutella laevigata	BLA	4.7± 1.2	2.93± 0.8	1	0.02± 0.003	0.12± 0.01
Centaurea linifolia	CLI	10.3± 0.6	0.75± 0.6	3	0.06± 0.01	0.21± 0.02
Centaurea paniculata	CPA	8.7± 1.6	12.92± 10	3	0.02± 0.003	0.13± 0.01
Cistus albidus	CAL	4.3± 1.5	5.33± 0.8	1	0.20± 0.02	6.75± 0.92
Cistus salvifolius	CSA	5.7± 1.5	0.21± 0.14	1	0.04± 0.01	5.8± 0.44
Convolvulus althaeoides	CON	8.3± 0.6	0.38± 0.1	1	0.07± 0.01	2.14± 0.16
Dorycnium hirsutum	DHI	9.0± 1.0	0.17± 0.2	3	0.08± 0.01	0.22± 0.03
Euphorbia flavicoma	EFL	1.3± 0.6	24.47± 11.7	1	0.01± 0.002	0.34± 0.03
Gallium aparine	GAP	11.7± 0.6	54.01± 47	1	-	0.1± 0.005
Gladiolus illyricus	GIL	4.0± 2.0	2.07± 0.9	2	0.22± 0.04	2.18± 0.30
Iris lutescens	ILU	4.0± 0.0	0.87± 0.7	2	-	8.98± 1.68
Leuzea conífera	LCO	12.3± 0.6	0.44± 0.2	3	0.04± 0.003	0.03± 0.004
*Linum strictum**	LST	9.0± 0.0	3.18± 3.4	2	0.003± 0.001	0.3± 0.01
Muscari neglectum	MNE	3.0± 1.7	0.39± 0.4	2	0.04± 0.003	2.39± 0.15
Orobanche latisquama	OLA	4.7± 0.6	2.25± 1.3	2	0.26± 0.04	0.83± 0.09
Phlomis lychnitis	PLY	9.7± 0.6	0.36± 0.1	3	0.53± 0. 05	0.49± 0.04
Ranunculus gramineus	RGR	3.0± 1.0	0.19± 0.1	1	-	8.89± 1.05
Rosmarinus officinalis	ROF	1.0± 0.0	487.64± 278.0	2	0.24± 0.02	0.91± 0.10
*Scorpiurus muricatus**	SMU	7.7± 0.6	0.77± 1.0	2	0.01± 0.002	0.58± 0.03
Sideritis hirsuta	SHI	4.7± 3.2	44.21± 33.6	3	0.04± 0.006	0.06± 0.005
Thymus vulgaris	TVU	1.7± 1.2	659.77± 244.1	2	0.02± 0.002	0.16± 0.04

Table 1. Beginning of bloom, flower density, corolla restrictiveness (1: open flower, 2: moderately restrictive; 3: highly restrictive), and nectar and pollen production per flower in the 23 plant species studied. All data are

based on measures from the 3 study years (2006, 2007 and 2008), except for species marked with (*), which did not bloom in 2006.

Pollinators showed large differences in biological traits. The earliest species appeared in the 1st week and the latest in the 14th. Body weight ranged from 0.0004 to 0.4 mg, and proboscis length between 0 and 21.07 mm.

1.3.3 Relationship between interaction strength and flower variables

The results of the ordination analyses relating interaction strength and flower features are represented in Figure 1. The first axis explained 15.2% of the observed species variance (Table 2) and was mainly related to flower density on the one hand and beginning of bloom on the other hand. The second axis explained 6.8% of the observed variance (Table 2) and was related to pollen and nectar per flower in opposition to corolla restrictiveness. The model including all variables was significant (p=0.012) and explained 31% of the observed variance. Of the 5 biological traits considered, flower density was significant and corolla restrictiveness marginally significant. Flower density (15% of explained variance; p=0.01) was primarily associated with the mass-flowering of *Rosmarinus officinalis* and *Thymus vulgaris* (Fig. 1b). Corolla restrictiveness (5% of explained variance; p=0.058) was mostly associated with composites with long, narrow tubular flowers such as *Centaurea linifolia*, *Centaurea paniculata* and *Leuzea conifera*, but also *Sideritis hirsuta* and *Dorycnium hirsutum* (Fig. 1b).

	1st axe	2nd axe	3th axe	4th axe
Eigenvalues	0.15	0.07	0.04	0.04
Cumulative percentage of variance of species data	15.2	22.0	26.3	30.0
Cumulative percentage of variance of species-environment relation	49.1	71.2	85.0	96.9
Sum of all canonical eigenvalues	0,31			

Table 2. Cumulative variance explained by RDA models relating flower traits and interaction strength.

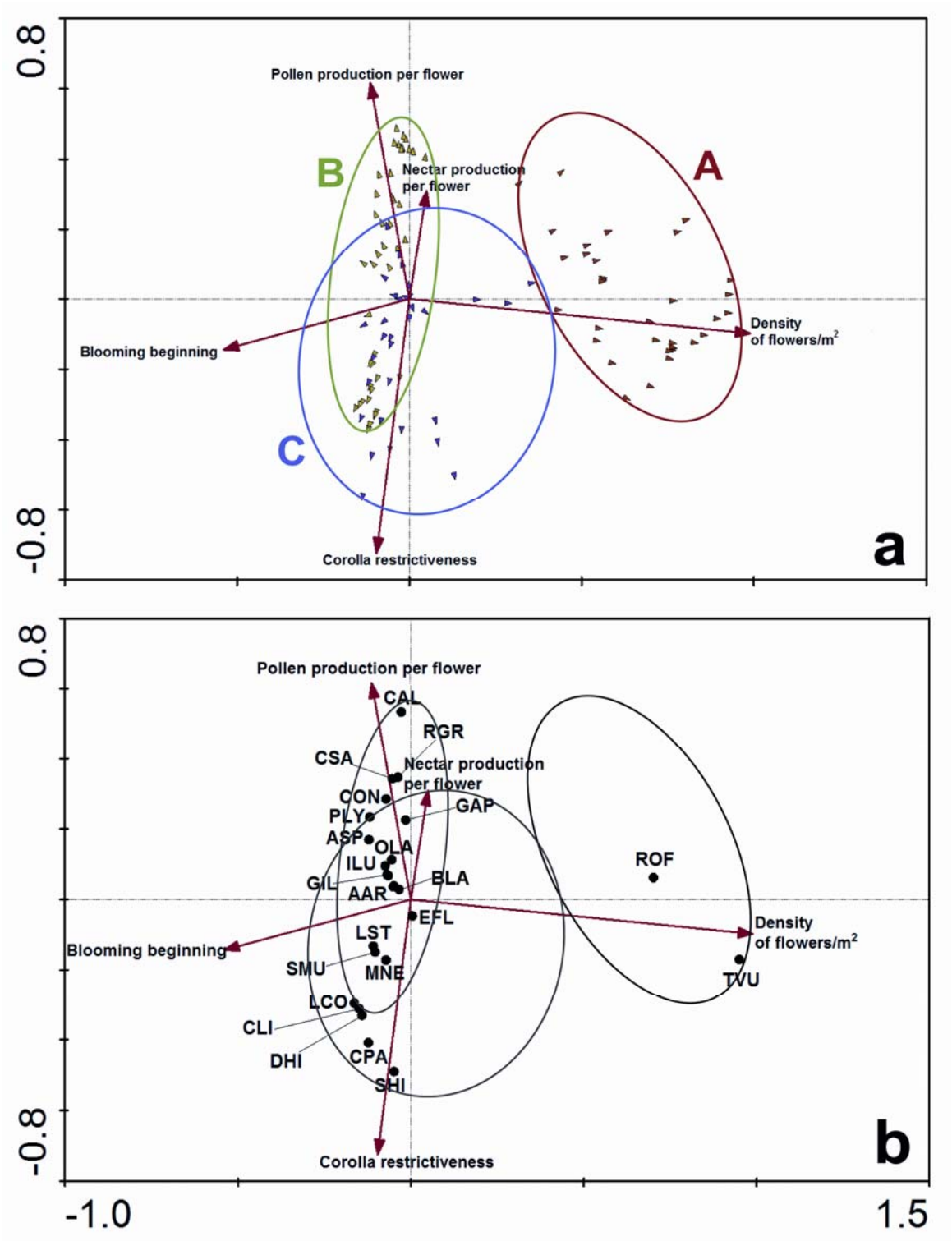

Fig. 1. Biplots of RDA relating flower variables and pollinator species (a) and flower variables and plant species (b). Pollinator groups (A, B, C) obtained from cluster analysis are shown in a. Plant species in b are indicated with acronyms (see table 1 for full names).

Since corolla restrictiveness is usually related to proboscis length, we further investigated the association between these two traits with a regression. For each pollinator species, we calculated a value of "preferred corolla restrictiveness" based on the mean restrictiveness value (1 to 3) of all the plant species the pollinator species visited, weighted by interaction strength. This value ranged from 1 (all visits to open flowers) to 3 (all visits to restrictive flowers). The presence of pollinator species with zero values of proboscis length (n= 30) caused problems with the data distribution. Thus, we finally removed these species from the analysis. We found a positive correlation between proboscis length and preferred corolla restrictiveness (n= 86; R^2= 0.17; $p<0.0001$). Pollinators with proboscis length equal to zero were mostly Coleoptera and small parasitic wasps. Preferred corolla restrictiveness for these species ranged from 1 to 2.91, indicating that short mouthparts are not restricting the use of restrictive flowers in our community.

We used a similar approach to further investigate the potential relationship between pollen/nectar production and pollinator body size. For each pollinator species we calculated a "preferred pollen and nectar per flower", based on the means of the pollen and nectar production of the flowers it visited, weighted by interaction strength. Body weight was not correlated with nectar per flower (n= 116; p=0.2), or pollen per flower (n= 116; p=0.06; R^2= 0.035).

1.3.4 Pollinator clusters and pollinator features

Cluster analysis based on the coordinates of pollinator species on the four first axes of RDA yielded three pollinator groups (Figs. 1 and 2). Cluster A included 35 species interacting mostly with plants with high flower density and early blooming period such as *R. officinalis* and *T. vulgaris* (Fig. 1). This cluster was mainly composed of female bees (12 species), and Diptera (13 species, mostly syrphids and beeflies) and did not include any Coleoptera (Fig. 3). The other two clusters (B and C) were distributed along the second RDA axis, defined by pollen and nectar production on the one hand and corolla restrictiveness on the other

(Fig. 1a). Cluster B was composed of 48 species mostly associated with flowers producing large amounts of pollen and nectar, and, weakly, with flowers with a late blooming period (Fig. 1). This cluster is largely represented by Coleoptera (20 species) and female bees (13 species) (Fig. 3). Finally, cluster C comprises 33 species showing a rather centred distribution in the RDA plot, but with a tendency to interact with plant species with restrictive corollas (Fig. 1). Cluster C is mostly represented by nectarivorous Hymenoptera (12 species) (Fig. 3).

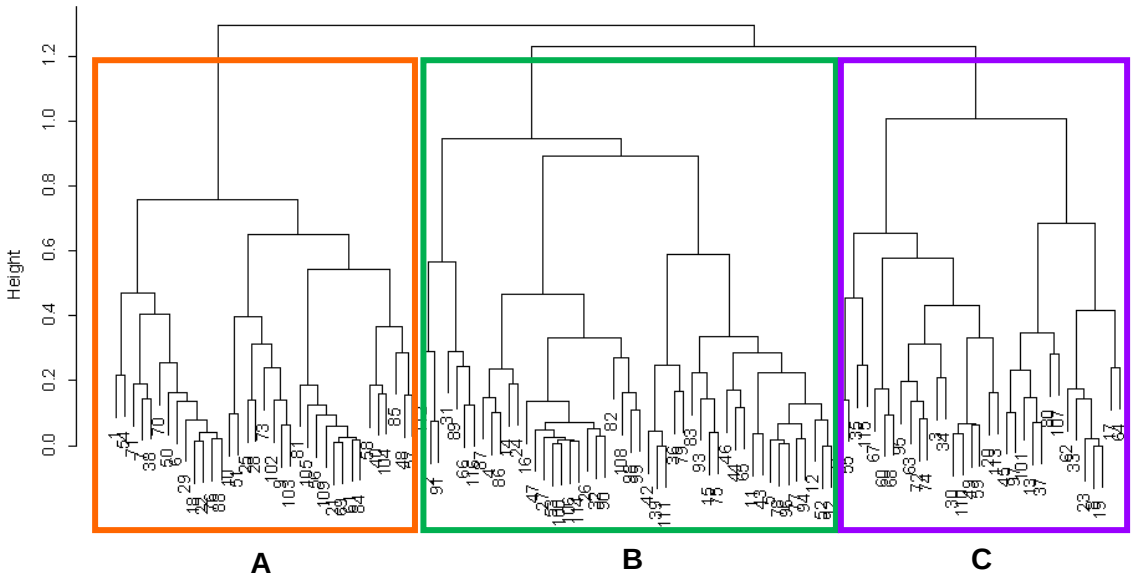

Fig. 2. Clusters of pollinator species (indicated by numbers) obtained from unpaired-group-method-analysis based on the coordinates of the four axes of the RDA in fig. 1.

Differences among pollinator clusters in activity periods were highly significant ($F_{2,113}$= 20.7; p <0.0001), with cluster A including the earliest pollinators and cluster B including the latest (Fig. 4). Differences in body weight were marginally significant (ANOVA, $F_{2,110}$= 2.92; p=0.058) (Fig. 4). According to post-hoc Tukey test, cluster A was composed of heavier species than cluster C (p=0.021), but differences between clusters A and B narrowly failed significance (p=0.08). Differences among clusters in proboscis length were significant (ANCOVA, $F_{2,110}$= 14.2; p<0.0001). According to the Tukey test, species of cluster B had shorter proboscis than species of cluster C (p<0.001) and A (p<0.001), and there

were no differences (p=0.74) between A and C (Fig. 4).

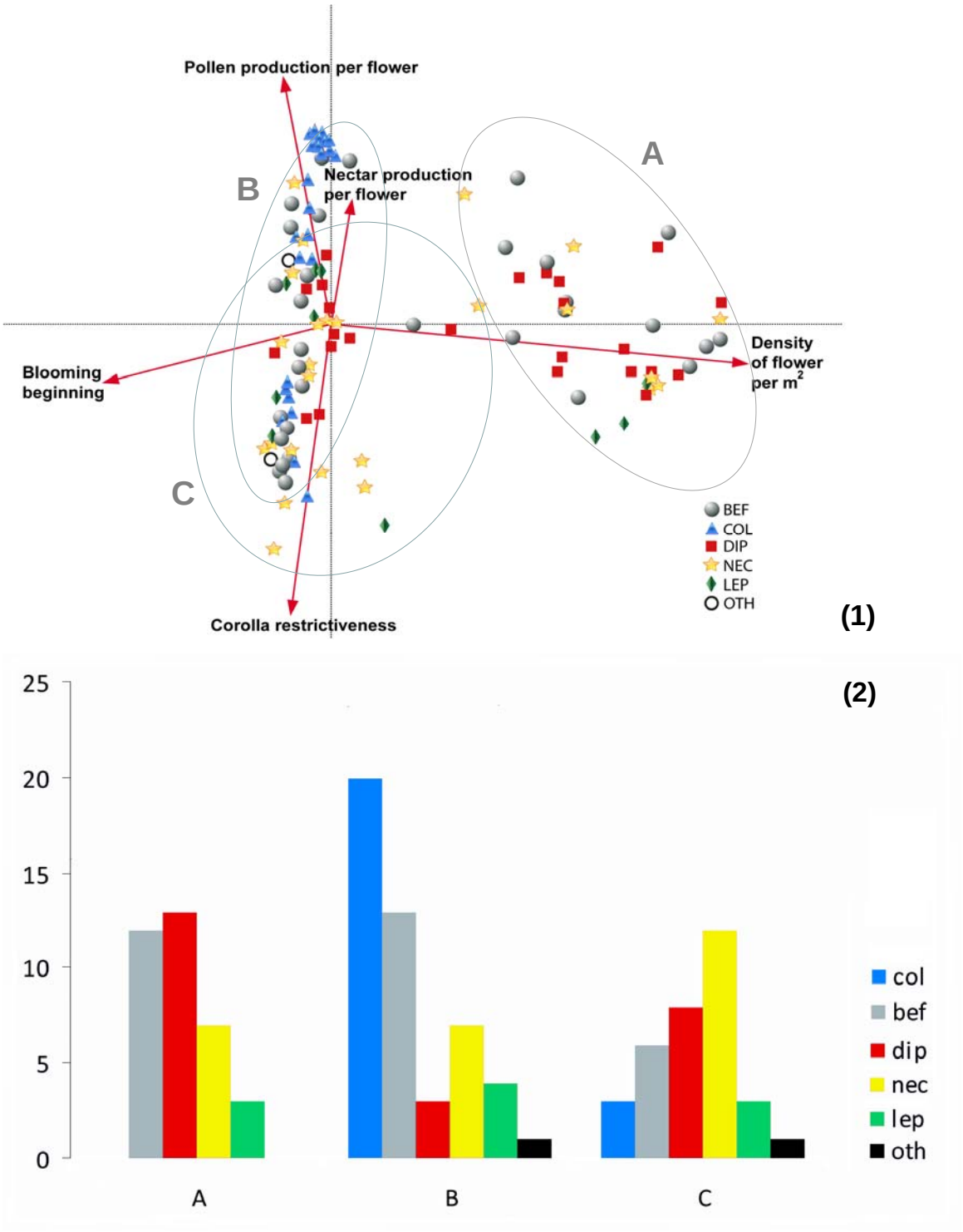

Fig. 3. Pollinator functional group distribution in the RDA biplot (1), and composition of the three resulting clusters (2). COL: Coleoptera; BEF: Female bees; DIP: Diptera; NEC: Nectar consumers; LEP: Lepidoptera; OTH: Others.

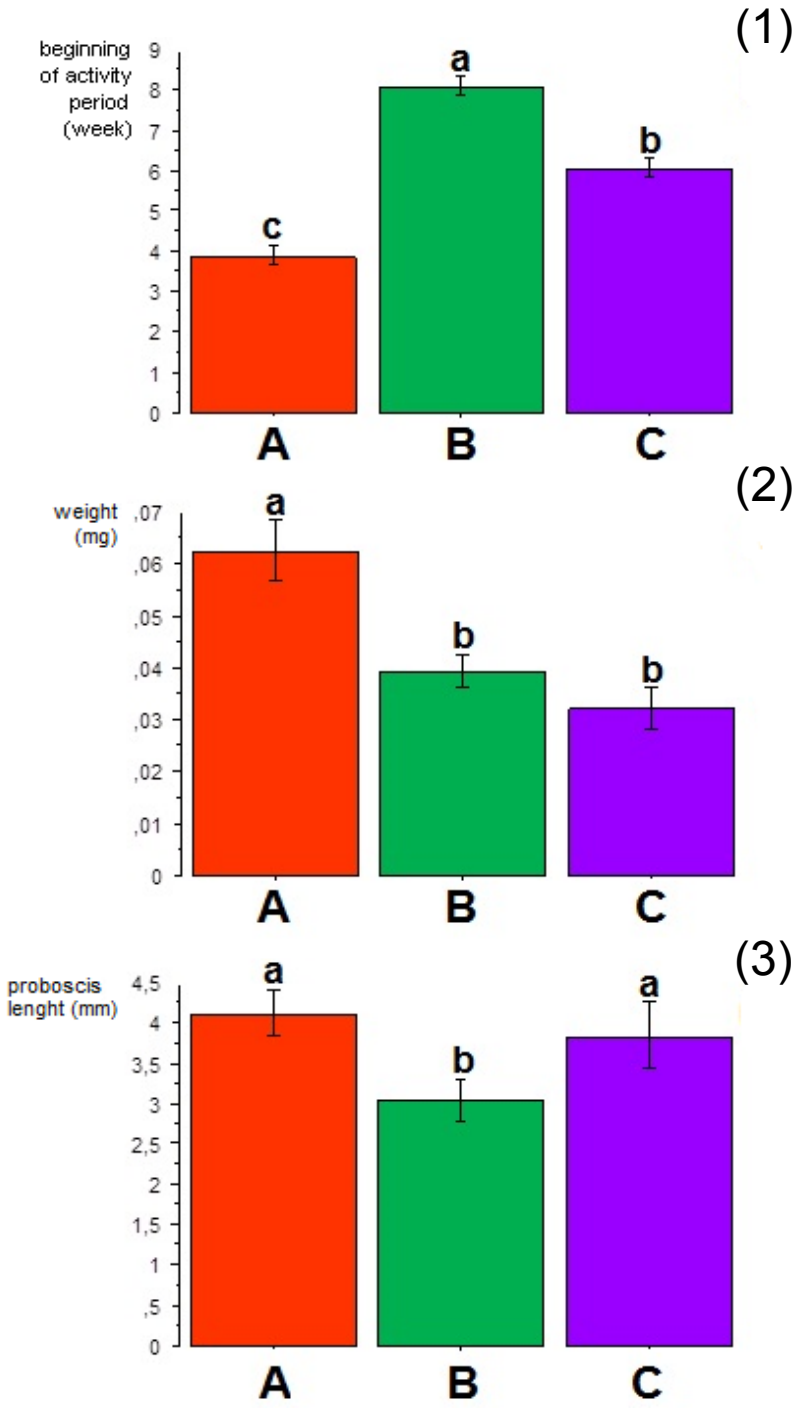

Fig. 4. Mean ± SE of beginning of activity period (1), proboscis length (2) and body weight (3) of the three pollinator clusters obtained from the RDA in fig. 1.

As expected, the effect of the covariate (weight) was significant ($F_{1,110}$= 86.6, p<0.0001). Interestingly, there was an interaction effect between cluster and wet weight ($F_{2,110}$= 3.84; p <0.04), indicating that the relationship between weight and proboscis length is different in different clusters.

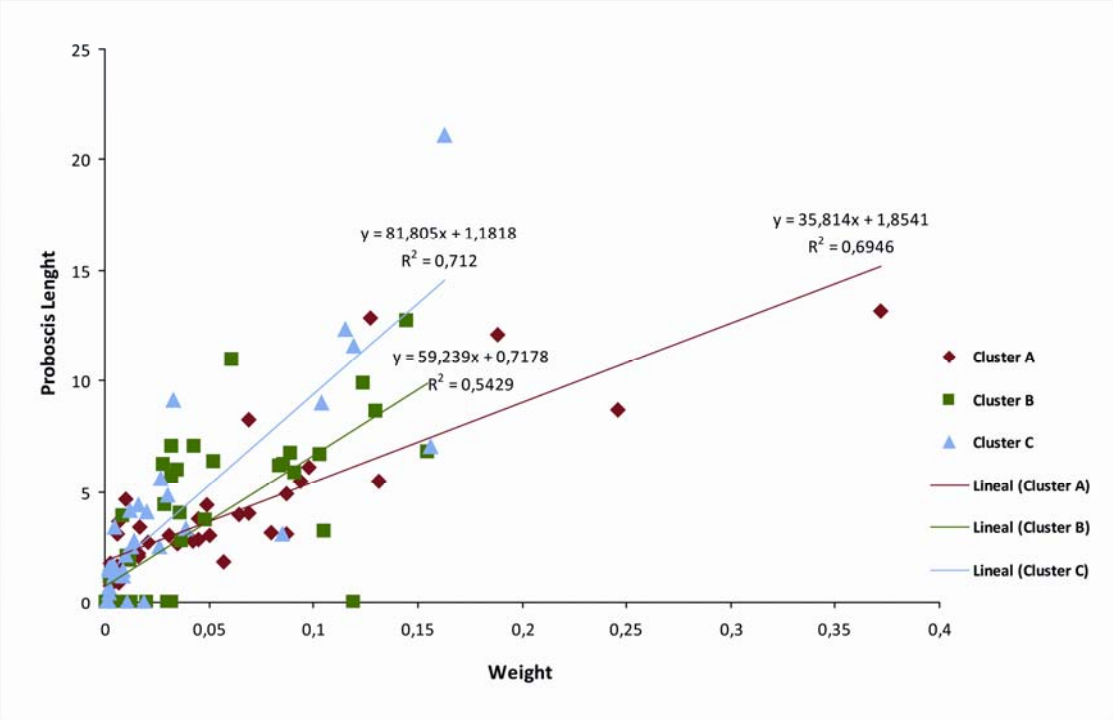

Fig. 5 Interaction effect between cluster and wet weight ($F_{2,110}$= 3.84; p <0.024) on proboscis length. The figure indicates that the relationship between weight and proboscis length is different in different clusters. In relation to their weight, pollinators of cluster C have longer proboscis (R^2=81.8) than pollinators of clusters A (R^2=35.8) and B (R^2=59.2).

In relation to their weight, pollinators of cluster C have longer proboscis (R^2=81.8) than pollinators of clusters A (R^2=35.8) and B (R^2=59.2) (Fig. 5).

Discussion

Previous studies in Mediterranean communities have found high levels of generalization in plant-pollinator interactions (C. M. Herrera 1988, J. Herrera 1988, Petanidou and Vokou 1990, Bosch et al. 1997, Petanidou et al. 2008), and our community is no exception (Bosch et al. 2009). Therefore, the moderate percentage (31%) of variance explained by our model is not surprising (for

example, see Potts et al. 2003). Most of this variance was explained by the axis opposing species with high flower density to species blooming late in the season. A lower proportion of the variance is explained by the axis opposing species with high pollen nectar/nectar production per flower to species with restrictive corollas.

The effects of flower density on pollinator visitation have been reported in both empirical and experimental studies, and at both population and community levels (Kunin 1997, Goulson et al. 1998, Bosch & Waser 1999, Thompson 2001, Mitchell et al. 2004, Hegland & Totland 2005, Feldman 2006, Dauber et al. 2010, Lázaro et al. 2013). In general, higher flower densities result in higher visitation rates, but this trend is not general, as high flower densities may result into forager saturation, thereby reducing visitation rates (Rathcke 1983, Essenberg 2013). Importantly, different pollinator groups have been found to respond differently to changes in flower density (Lázaro & Totland 2010). Therefore, an ecological traits (flower density) appears to override the importance of biological attributes in structuring plant-pollinator interactions in our community. Two characteristics inherent to the community studied contribute to explain this result. First, the Garraf flower community is largely dominated by two species, *R. officinalis* and *T. vulgaris*, which together account for 76% of the flowers counted in transects (Flo 2014). As many as the 24% of the remaining 21 plant species studied contribute less than 1% of the community's flower production. As a consequence, differences among species in flower density are much greater than differences in any of the biological attributes studied (Table 1). Second, the Garraf plant-pollinator community is strongly seasonal, with a quick turnover of flower composition. In addition to their high flower density, *R. officinalis* and *T. vulgaris*, also bloom early in the season, and are associated with cluster A, mostly composed of early-flying pollinator species. Seasonality is characteristic of Mediterranean systems (J. Herrera 1988, Petanidou et al. 1995, Bosch et al. 1997, Bosch et al. 2009, Filella et al. 2013), and affects not only flowering periods but also activity periods of pollinators, which then two be short thus resulting in important temporal changes in pollinator composition. In our study, and in

agreement with results of other Mediterranean communities (J. Herrera 1988, Bosch et al. 1997), Coleoptera did not appear until the month of April and were totally absent from cluster A.

Although to a lesser extent than flower density, corolla restrictiveness also emerged as a floral trait structuring plant-pollinator interactions. According to optimal foraging theory, variability in corolla depth and proboscis length should lead to resource partitioning: nectar feeders with long proboscides should specialise in flowers with deep corolla tubes, and nectar feeders with short proboscides in shallow flowers (Rodríguez-Gironés & Santamaria 2006; 2010). Some studies have partially confirmed this assumption (Sakai et al. 1999). It is especially well documented in bumblebees (Inouye 1980, Ranta & Lundberg 1980, Dohzono et al. 2004, Peat et al. 2005), but also in butterflies (Corbet 2000). In our community, plants with restrictive corollas were weakly associated with cluster C, mostly represented by nectarivorous Hymenoptera and comprising species with long proboscis (in absolute terms and in relation to their body size). Other studies have found a positive relationship between corolla depth and nectar production (Petanidou & Smets 1995), but this two traits appear to be opposed in our community. Therefore, the relationship between corolla shape and nectar-feeding Hymenoptera in our community seems to be based on nectar accessibility rather than on nectar production. Corolla restrictiveness is believed to have evolved as a barrier to avoid inefficient pollinators (Laverty 1980, Castellanos et al. 2004, Rodríguez-Gironés & Santamaría 2007) and several studies have shown flowers with restrictive corollas to have narrower arrays of flower visitors (Fenster 1991, C. M. Herrera 1996, Fenster et al. 2004, Stang et al. 2006). A confounding factor in the relationship between corolla restrictiveness and proboscis length has to do with pollen accessibility. Some pollinator groups, notably Coleoptera, collect mostly pollen. Although mostly associated with species with high levels of pollen production per flower, this group is also found on flowers with restrictive corollas (Fig. 3). The species with the most restrictive corollas in our community are *Centaurea linifolia*, *Centaurea paniculata* and

Leuzea conifera. However, in these species anthers grow out of the corolla aperture and therefore pollen is readily accessible and actively collected by Coleoptera. This explains the lack of relationship between restrictiveness preference and proboscis length when pollinators with proboscis length equal to zero (mostly Coleoptera) are included.

Floral rewards did not emerge as important drivers of plant-pollinator interaction structure. However, cluster B, mostly composed of species collecting large amounts of pollen (Coleoptera and female bees), was weakly associated with plants producing large amounts of pollen per flower. Mediterranean plants typically produce low amounts of nectar, and a major role of pollen seems to be a common outcome in Mediterranean communities (J. Herrera 1988, Petanidou & Smets 1995, Petanidou & Lamborn 2005). Some studies however, have found different results. In a western Mediterranean grassland community in which rewards and morphological floral traits were more important than seasonality in explaining pollinator partitioning, large bees were associated with plants producing large amounts of nectar (Bosch et al. 1997). In an eastern Mediterranean mosaic landscape, bee diversity was associated to nectar diversity and to high pollen/nectar ratio, rather than to any measure of nectar or pollen abundance Potts et al. (2003, 2004).

According to pollination energetics theory (Heinrich 1975), a relationship between floral rewards and pollinator energetic requirements (expressed as body size) should be expected. We did not find such a relationship. To our knowledge, empirical support for this kind of relationship has only been reported in comparisons of pollinators differing in body size by many orders of magnitude (e.g. insects *versus* birds; Brown et al. 1978, Kodric-Brown et al. 1984, Dalsgaard et al. 2009). Other studies have found that nectar-collecting bumblebees of different sizes select different flowers, but this appears to be mostly the result of a positive relationship between body size and proboscis length and its association to corolla tube depth, rather than a direct relationship between body

size and amount of reward (Heinrich 1976, Inouye 1978, Ranta & Lundberg 1980, Pleasants 1983). Many factors may hinder a direct relationship between body size and reward. First, pollinators may be responding to different reward currencies. For example, large pollinators may increase their net energetic gain by favouring species with large amounts of reward per patch, rather than per flower, thus minimizing energetic expenditure in flights between flowers (Heinrich 1975). However, studies exploring this possibility have not found a direct relationship between body size and patch size (Stout 2000, Tschapka 2004). Second, even if large pollinators could only profitably forage on flowers with large amounts of rewards, small pollinators should be able to satisfy their energetic needs both in flowers producing small and large amounts of rewards. Third, flowers producing large amounts of rewards also attract more pollinators (Bosch 1992). This may have an equalizing effect across the species in the community, tending to a situation of ideal free distribution (Fretwell & Lucas 1970, Dreisig 1985).

The Garraf plant-pollinator community is structured in three clusters. The most distinct of these clusters is composed of early-flying species (mostly female bees and Diptera) of large body size and with long mouthparts. This cluster is associated with the two plant species dominating the flower community (*R. officinalis* and *T. vulgaris*), both of which bloom early. We believe this association is mostly dictated by phenology. The few pollinator species active early in the season visit the few flower species available, which happen to produce large amounts of flowers. This flowering pattern early in the season has been interpreted as a strategy to attract pollinators at a time when these are scarce (Filella et al. 2013). Mass-flowering could also be related to large body size, as large pollinator species, with high energetic requirements and high cost of flight, would be expected to favour abundant flower species, thus maximizing net energy gain (Heinrich 1975). However, large body size at the beginning of the season may be an inherent characteristic of the pollinator community. Large bee species in Mediterranean communities have been found to start their activity

early in the season when temperatures are still low (Osorio et al. 2015), probably in relation to their better thermoregulatory capacity (Stone & Willmer 1989, Heinrich 1993, Bishop & Armbruster 1999).

With the decline of *R. officinalis* and *T. vulgaris*, the flower-pollinator ratio is reversed, there are much fewer flowers available and visitation rates per flower increase dramatically (Bosch et al. 2009, Filella et al. 2013). At this time, the relationships between floral and pollinator traits are expected to become more diffuse as pollinators cannot afford to be choosy. The two clusters corresponding to mid and late season are in fact more centred in relation to floral traits. Cluster B is mostly composed of Coleoptera and female bees, and is weakly associated to plants producing large amounts of pollen per flower. Cluster C is dominated by nectarivorous Hymenoptera and is weakly associated to flowers with restrictive corollas. These two clusters have again a seasonal component, with cluster C corresponding to the mid season and cluster B to the late season.

Our results do not provide strong support for the pollination syndrome theory (Proctor et al. 1996). Most plant species in our community are wide generalists (Bosch et al. 2009), and pollinator attraction seems to be more dependent on pollinator traits than on taxonomic status. Phenology and flower density were more important than classical floral traits in explaining plant-pollinator interactions. As opposed to tropical communities (CM Herrera 1996), differences among plants in reward production and flower morphology may be too small in Mediterranean communities to elicit large differences in pollinator assemblages attracted. Under these circumstances, the appearance of well-defined pollination syndromes is unlikely (Wilson et al. 2004) and any effect of floral traits may be overridden by ecological traits and seasonal patterns.

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Chapter 2

Floral advertisement scent in a changing plant-pollinators market

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Floral advertisement scent in a changing plant-pollinators market

Abstract

Plant-pollinator systems may be considered as biological markets in which pollinators choose between different flowers that advertise their nectar/pollen rewards. Although expected to play a major role in structuring plant-pollinator interactions, community-wide patterns of flower scent signals remain largely unexplored. Here we show for the first time that scent advertisement is higher in plant species that bloom early in the flowering period when pollinators are scarce relative to flowers than in species blooming later in the season when there is a surplus of pollinators relative to flowers. We also show that less abundant flowering species that may compete with dominant species for pollinator visitation early in the flowering period emit much higher proportions of the generalist attractant β-ocimene. Overall, we provide a first community-wide description of the key role of seasonal dynamics of plant-specific flower scent emissions, and reveal the coexistence of contrasting plant signaling strategies in a plant-pollinator market.

2.1. Introduction

Many plants produce rewards in the form of nectar and pollen that attract pollinators, thus ensuring the transfer of pollen from flower to flower. Plant-pollinator communities may thus be considered as biological markets in which pollinators choose between different flowers that may compete for their visits (Cohen & Shmida 1993, Noe & Hammerstein 1995, Chittka & Schürkens 2001). Flowers rely on sensory signals to advertise their rewards, color and scent being the most important ones (Chittka & Raine 2006). Historically, plant–pollinator relationships have mostly been considered as a visually-mediated process, and

floral odors have received less consideration (Raguso 2008). However, olfactory cues are often the basis upon which pollinators make flower choices, because scent cues are easily learned and remembered by pollinators (Wright & Schiestl 2009). Different studies have revealed that bees are able to detect pollen and nectar in flowers via odour cues (Dobson 1987, Dobson & Bergström 2000, Goulson et al. 2001, Howell & Alarcon 2007, Wright & Schiestl 2009 and references therein), that bees learn odours faster and remember them for longer than visual cues (Menzel 1991, Wright & Schiestl 2009), that specific pollen odour plays a key role in host recognition by oligolectic solitary bees (Dobson 1987, Dobson & Bergström 2000), and that floral odour differences are important for maintaining reproductive isolation between closely related plant species (Waelti et al. 2008). Other studies have revealed that plant and floral scents elicit a foraging response also in other insect pollinators (Cook et al. 2002, Andersson 2003, Primante & Dötterl 2010). In addition, floral scent has been found to improve plant fitness via increased pollinator attraction (Majetic et al. 2009a). Nevertheless, in spite of the putative importance of flower odors in structuring plant-pollinator interactions, community-wide patterns of flower scent signals and their seasonal dynamics remain largely undescribed.

As in most markets, supply and demand in plant-pollinator systems fluctuate in time. Certain periods are characterized by a surplus of flowers relative to pollinators, which may result in competition between flowers and large investment in rewards and display (Cohen & Shmida 1993). Conversely, in periods exhibiting a surplus of pollinators relative to flowers, a reduction of investment in floral rewards and display is expected. In the Mediterranean region, the peak of flowering occurs in the early spring (March-April) and hot, dry summers present a physiological challenge to plants (Kummerov 1983, Petanidou et al. 1995, Bosch et al. 1997). The early flowering peak results in a surplus of flowers relative to pollinators in spring, followed by a surplus of pollinators in relation to flower availability in summer (Cohen & Shmida 1993).

We studied a plant-pollinator community in a Mediterranean shrubland, in which flower and pollinator availability follow closely this model of a seasonal floral market (Bosch et al. 2009). To explore the existence of contrasting plant signaling strategies, we quantified floral scent compounds for each plant species as well as the seasonal variation of flower abundance, nectar and pollen availability, and flower visitation rates. Specifically, we examined two hypotheses associated with the emergence of differentiated plant-signaling strategies in plant-pollinator networks. Firstly, we hypothesized a greater investment in scent advertisement early rather than late in the flowering period associated with the lower pollinator availability (pollinator abundance hypothesis). Secondly, we examined whether less abundant flowering species, which, other factors being equal, might have difficulty attracting pollinators, produce a different scent from abundant species (plant abundance hypothesis). We tested these two hypotheses and provide a first integrative description of community-wide patterns of flower scent signals.

2.2. Materials and methods

2.2.1. Study area and Field surveys

The study was conducted in a Mediterranean shrubland community in Garraf Natural Park (Barcelona, NE Spain), 340 m above sea level and 1700 m from the coastline. Field work was conducted in a ca. 1ha plot, from late February to late June, encompassing the main flowering period in the area. No plants were in bloom during the dry summer season (July–August). In 2008, we counted weekly the number of open flowers in six 50 x 1 m transects and conducted pollinator counts on 24 plant species, representing 99.96% of the total number of flowers in the study plot: *Rosmarinus officinalis, Thymus vulgaris* (hermaphrodite and female morphs), *Muscari neglectum, Ranunculus gramineus, Euphorbia flavicoma, Iris lutescens*, *Biscutella laevigata Cistus salvifolius, Dorycnium hirsutum, Cistus albidus, Orobanche latisquama, Gladiolus illyricus, Galium aparine, Scorpiurus muricatus, Anagallis arvensis, Convolvulus althaeoides,*

Centaurea linifolia, Centaurea paniculata, Sideritis hirsuta, Phlomis lychnitis, Linum strictum, Leuzea conifera and *Allium sphaerocephalon*. Floral rewards were measured on 15-20 flowers of each species. To measure volume of pollen produced per flower, we estimated the number of pollen grains in undehisced anthers in a 70% ethanol-pollen suspending solution using an electronic particle counter (Coulter Multisizer), and measured pollen grain size under the microscope. To measure nectar production (mg of sugar produced per flower) we bagged flower buds and 24 h following anthesis, we used micropipettes to extract the accumulated nectar. Sugar concentration was measured with field refractometers.

Pollinator surveys were conducted twice a week throughout the blooming period. Flower patches were tagged, open flowers were counted and observed for 4 min periods throughout the day. During the observation time insects visiting the flowers were visually identified, and contacts were counted. Pollinators that could not be identified in the field were captured for later identification. From pollinator surveys, we obtained a measure of pollinator visitation rates (visits per flower and unit time).

2.2.2. Floral BVOC (biogenic volatile organic compounds) emission rates

We sampled the emission of flowers from 5 individuals of each plant species in its peak flowering week in 2009. Additionally, to test whether floral scent emission throughout the season could be the result of phenotypic plasticity, we sampled flowers from 5 individuals of 5 plant species (*R. officinalis, E. flavicoma, M. neglectum*- species flowering mainly early in the season-, *B. laevigata* – flowering the whole season- and *P. lychnitis*-flowering late in the season) throughout their entire blooming period in 2011. In both cases samples were taken in the field at midday. We carefully put our specimens in water vials and immediately transferred them to a portable 4ºC cabinet prior to analyses with gas chromatography (GC-MS) and Proton Transfer Reaction Mass Spectrometry (PTR-MS). BVOC analyses, with special focus on isoprenoids, were performed

through head space technique in the GC-MS (Agilent Technologies, GC: 7890A, MS: 5975C inert MSD with Triple-Axis Detector, Palo Alto, CA, USA). In the laboratory, flowers (inflorescences in the case of *Centaurea* spp. and *Leuzea conifera*) were separated from vegetative parts. This procedure was applied for each of the 10 individuals of each plant species. Flowers were introduced in 10 ml vials which were then placed 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). Incubation time was 10 min. at 35°C. Two ml samples were injected into a 30m x 0.25mm x 0.25mm film thickness capillary column (HP-5MS, Agilent Technologies). Helium flow was 0.5 ml min^{-1}. Total run time was 30 min. and the solvent delay was 4 min. After the sample injection, the initial time was 1 min. and the initial temperature (40 °C) was increased at 15 ºC.min^{-1} up to 150 °C and kept for 5 min, and thereafter at 50°C.min^{-1} up to 250 ºC where the temperature was kept for 5 min., and thereafter at 30°C.min^{-1} up to 280 °C, which was maintained for 5 min. The identification of monoterpenes was conducted by comparing retention times with liquid standards from Fluka (Buchs, Switzerland) volatilized in the vial, and the fractionation mass spectra with standards spectra and Nist05a and wiley7n mass spectra libraries. Terpene concentrations were determined using calibration curves for common monoterpenes, alpha-pinene, beta-pinene, 3-carene, linalool, and sesquiterpene alpha-humulene. The analyses of emission rates for all emitted volatiles were conducted with a PTR-MS. Flowers were enclosed in a leaf cuvette of a LCpro+ Photosynthesis System (ADC BioScientific Ltd., Hoddesdon, England) at 25ºC, and the air exiting the leaf cuvette was monitored with flow meters and analyzed with a Proton-Transfer-Reaction Mass Spectrometer (PTR-MS-FTD hs) from Ionicon Analytik, Innsbruck, Austria. These VOC analyses were replicated three times for each sample. The quantification of VOCs was based on the use of replicated three times calibration standards (ethylene, methanol, isoprene, alpha-pinene, methyl salicylate and caryophyllene, Sigma-Aldrich, Abelló- Linde). The PTR-MS drift tube was operated at 2.1 mbar and 40ºC, with a drift field of 600 V cm^{-1}. The

parent ion signal was maintained at around $3x10^6$ counts per second during the measurements. We conducted scans of all masses between 22 and 205 to determine which compounds were emitted by the different samples (Peñuelas et al. 2005). Previous to any measurement, we measured the background concentrations of VOCs in the empty cuvette, and considered these data to calculate the emission/uptake of every compound.

We estimated emission rates at the field temperature by using the equation
M = MTS exp(b (T-Ts)) (Guenther et al. 1993) where M is the emission rate at temperature T, MTS is emission rate at 303 K, b is an empirical coefficient and Ts = 303 K.

2.2.3. Data analysis

To test for differences between early and late-blooming plants in total BVOC emission, flower abundance, nectar and pollen content and pollinator abundance we conducted permutational multivariate ANOVAs (PERMANOVA) (Anderson et al. 2008) using the Bray Curtis index of similarity, with “season” (early flowering period, late flowering period) as a fixed factor. We also conducted a cluster analysis on the percentage of the different VOCs emitted by each species. All these analyses were conducted using the statistical packages PERMANOVA+ for PRIMER v.6 (Anderson et al. 2008) and Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA). We also used the program PHYLOMATIC (Webb & Donoghue 2005) to build a phylogenetic tree of the plant species studied and test if total terpene emission showed a significant phylogenetic signal- i.e. the tendency of closely related species to resemble each other due to shared ancestry- as described in Peñuelas et al. 2010. Briefly, PHYLOMATIC uses a backbone plant megatree based on a variety of sources involving primarily DNA studies to assemble a phylogenetic tree for the species of interest. Our phylogenetic hypothesis was based on the conservative megatree, where unresolved nodes were included as soft polytomies. We used the PDAP package (Garland et al. 1993) to transform the phylogenetic tree into a matrix of phylogenetic distances, and tested if the

studied traits showed significant phylogenetic signal with the randomization procedure in the PHYSIG module developed by Blomberg et al. 2003. This test compares the variance in phylogenetic independent contrasts observed in the real dataset against a null distribution obtained when the phenotypic data are randomized across the tips of the tree (breaking any pattern of phylogenetic resemblance between relatives). Phylogenetic signal was considered significant if the variance in contrasts of the real dataset was lower than the variance in 95% of the permuted datasets. These analyses were performed to determine if phylogenetic correction was necessary in subsequent regression analyses. When the dependent variable showed significant phylogenetic signal we used phylogenetic generalized least square regressions (PGLS). PGLS controls for phylogenetic relatedness by adjusting the expected variance/covariance of regression residuals using the matrix of phylogenetic distances (this approach is mathematically equivalent to analyzing the data with phylogenetically independent contrasts). These analyses were performed with the REGRESSIONV2 module in MATLAB 7.6.0 (Lavin et al. 2008). We used the stats package R Core Team to draw the heatmap of volatile emissions in each species.

2.3. Results

The overall flowering period extended from late February to June. The community presented a clear seasonal pattern with two contrasting scenarios. Early in the flowering period, from late February until early April, flower and floral reward availability (nectar and pollen) was high and visitation rates (pollinator visits per flower and unit time) low. On the other hand, from mid April until June, flower and floral reward availability were much lower, and pollinator visitation rates were much higher (Fig. 1). Plant species were therefore divided in two groups (early and late flowering species) using as a criterion the time when the drastic decline in flower availability coincided with a drastic increase in pollinator visitation (Fig. 1). The species with their peak of flowering early in the season

(flowering from late February to early April) were *Rosmarinus officinalis, Thymus vulgaris* (hermaphrodite and female morphs), *Muscari neglectum, Ranunculus gramineus, Euphorbia flavicoma* and *Iris lutescens*. The species with their peak of flowering in the second half (flowering from early April to June) were *Cistus salvifolius, Dorycnium hirsutum, Cistus albidus, Orobanche latisquama, Gladiolus illyricus, Galium aparine, Scorpiurus muricatus, Anagallis arvensis, Convolvulus althaeoides, Centaurea linifolia, Centaurea paniculata, Sideritis hirsuta, Phlomis lychnitis, Linum strictum, Leuzea conifera* and *Allium sphaerocephalon*. One especies, *Biscutella laevigata,* was in bloom during most of the flowering season (March to June) and was not included in the analyses. Bees were the main flower visitors until the end of may (see Appendix Fig. S1).

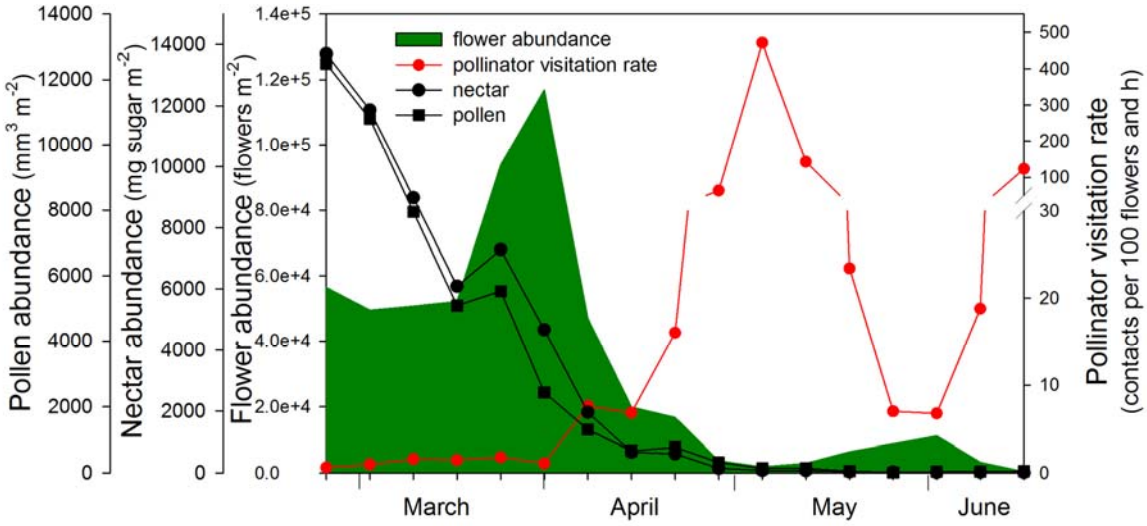

Fig. 1 Seasonal pattern of weekly flower abundance (number of flowers per m²), flower rewards (nectar and pollen, mg sugar and mm³ of pollen volume per m²), and pollinator visitation rate (number of pollinator contacts per 100 flowers and h) in the Garraf plant-pollinator community in 2008. Note the break in the axis of the pollinator visitation rate. This seasonal pattern was consistent between years (authors' observation during the period 2006-2009).

The floral scent of species flowering early in the season significantly differed from the floral scent of species flowering later (pseudo-$F_{1,22}$ = 8.06, P<0.001, PERMANOVA, see Appendix Table S1-part 1 and 2- and Fig. S2). Species flowering early emitted higher amounts of terpenes per flower and per dry weight

of flower ($F_{1,21}$ = 12.8 P<0.01, $F_{1,21}$ = 6.03, P<0.05, respectively, n=23 species; Fig. 2a).

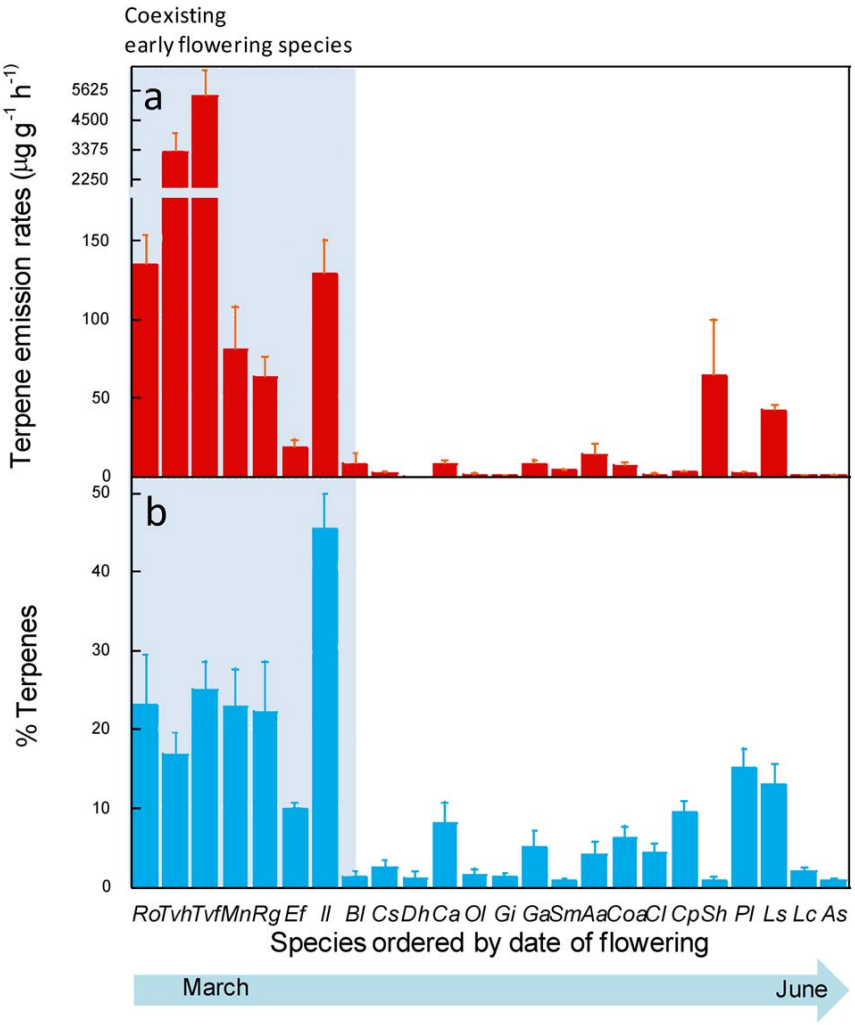

Fig. 2 Pollinator abundance hypothesis. Seasonal pattern of (a) terpene emission rates and (b) percentage of terpenes emitted relative to the total emission of biogenic volatile compounds by the plant species of the Garraf shrubland community ordered by date of flowering peak. Note the break in the axis for the terpene emission rates. Early (from late February to early April): *Ro- Rosmarinus officinalis, Tvh- Thymus vulgaris* hermaphrodite*, Tvf- Thymus vulgaris* female, *Mn- Muscari neglectum, Rg- Ranunculus gramineus, Ef- Euphorbia flavicoma, Il- Iris lutescens;* late (from early April to June): *Cs- Cistus salvifolius, Dh- Dorycnium hirsutum, Ca- Cistus albidus, Ol- Orobanche latisquama, Gi- Gladiolus illyricus, Ga- Galium aparine, Sm- Scorpiurus muricatus, Aa- Anagallis arvensis, Coa- Convolvulus althaeoides, Cl- Centaurea linifolia, Cp- Centaurea paniculata, Sh- Sideritis hirsuta, Pl- Phlomis lychnitis, Ls- Linum strictum, Lc- Leuzea conifera, As- Allium sphaerocephalon. Bl- Biscutella laevigata* blooms during most of the flowering season (March to June). Error bars are SE (n=5).

After correcting emissions by field temperatures, species flowering early still emitted higher amounts of terpenes (per flower and per dry weight of flower) ($F_{1,21}$ = 9.23 P<0.01, $F_{1,21}$ = 5.15 P<0.05, respectively, n=23 species). These species also emitted a higher proportion of terpenes relative to total volatiles ($F_{1,21}$ = 34.2, p<0.0001, ANOVA, Fig. 2b). There was not any significant phylogenetic signal in these differences for terpene emissions (p=0.80) (see Appendix Fig. S3).

Among the species flowering early in the flowering period, *Rosmarinus officinalis* and *Thymus vulgaris* largely out-numbered the rest of species in number of individuals and number of flowers per individual (Fig. 3). These two species accounted by far for most of the nectar and pollen produced during this period (Bosch et al. 2009). Their scent was different from that of less abundant co-flowering species (pseudo-$F_{1,6}$ = 12.27, P=0.001). Notably, these less abundant species co-flowering with *R. officinalis* and *T. vulgaris* emitted a similar flower fragrance with a very high proportion of the monoterpene β-ocimene (Fig. 3). The percentage of β-ocimene emissions after controlling for phylogenetic relatedness was still higher in the less abundant species than in *R. officinalis* and *T. vulgaris* (p=0.019, n=5, PGLS, phylogenetic generalized least square regressions). Early flowering species shared pollinators (see Appendix Fig. S1b), most of which were generalists in their flower-visiting habits (see Appendix Fig. S1).

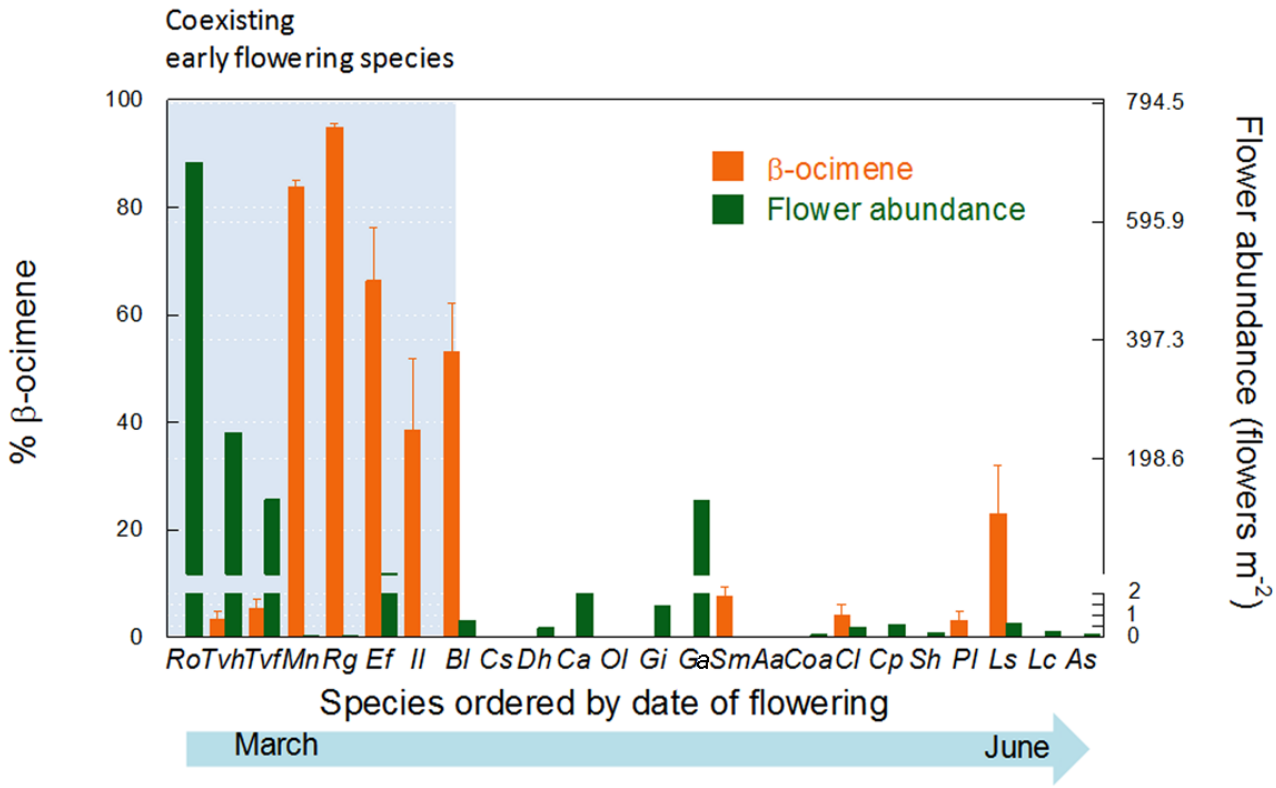

Fig. 3 Plant abundance hypothesis**.** Flower abundance (number of flowers per m^2) and percentage of β-ocimene emitted relative to the total emission of terpenes by the plant species of the Garraf shrubland community ordered by date of flowering peak as described in Figure 2. Note the break in the axis for the flower abundance. Note that although peaking in the second half of the season, *Bl- Biscutella laevigata* overlaps with the early flowering species throughout March and April. Error bars are SE (n=5).

No significant seasonal trend was found in the emission rates of total terpenes, nor in the emission rates of β-ocimene in particular, in any of the five species studied throughout their entire blooming period (*R. officinalis, E. flavicoma, M. neglectum*- flowering mainly early in the season-, *B. laevigata* –flowering the whole season- and *P. lychnitis*-flowering late in the season) (Fig. 4).

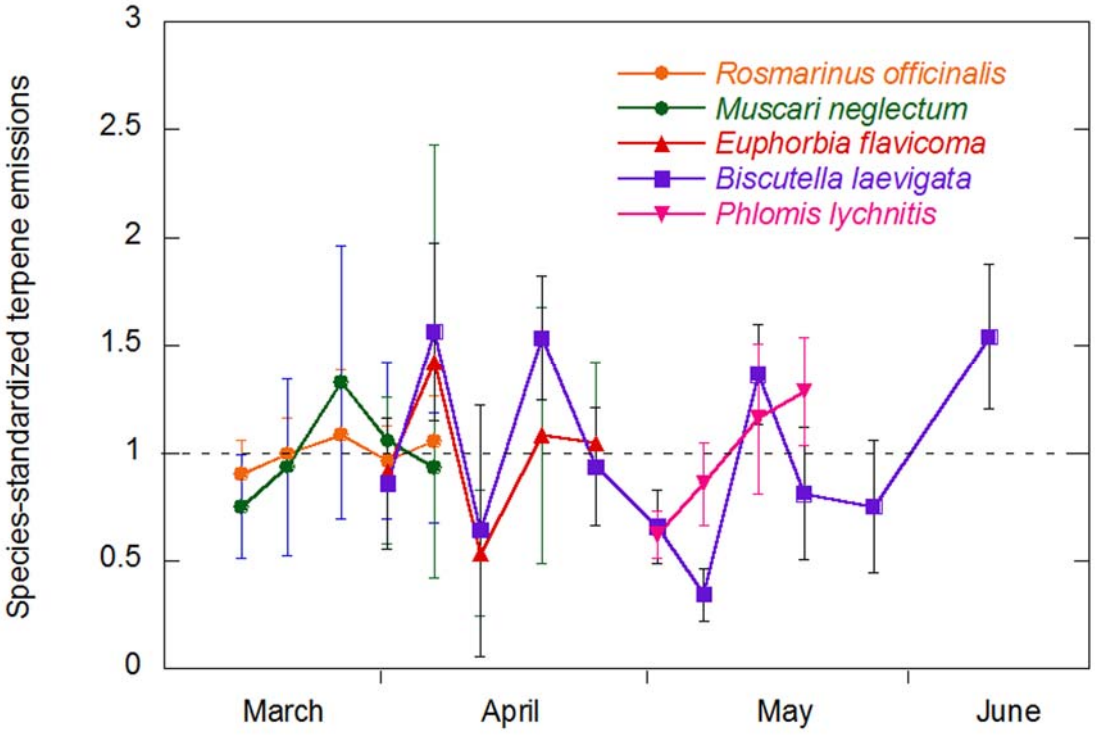

Fig. 4 Species-standardized terpene emission rates (μg g^{-1} h^{-1}) (emission rates were divided by the mean emission rate of each species) of five representative species throughout their entire blooming period. Error bars are SE (n=5). Species standardized β-ocimene emission rates also followed no particular pattern (data not shown).

2.4. Discussion

In accordance with the pollinator abundance hypothesis, we found that species flowering early in the season presented a higher scent emission than plant species flowering later, with the former emitting a higher amount and proportion of terpenes. Floral scents dominated by terpenes are common among plants pollinated by bees (Dobson 2006), the main pollinator group during the early flowering period in this community (see Appendix Fig. S1). Moreover, terpenes emissions have been suggested to be major contributors to the effect of floral scent emissions on seed fitness (Majetic et al. 2009a).

As expected based on the plant-abundance hypothesis, we found that *Rosmarinus officinalis* and *Thymus vulgaris* scent was different from that of less

abundant co-flowering species, with the less abundant species emitting a similar fragrance dominated by β-ocimene. β-ocimene is known to be a general attractant, emitted by a wide range of plants pollinated by different groups of pollinators (Knudsen et al. 2006), such as bees (Gerlach & Schill 1991, Borg-Karlson et al. 1994), moths (Knudsen & Tollsten 1993, Okamoto et al. 2007), butterflies (Andersson et al. 2002), and beetles (Dufaÿ et al. 2003, Okamoto et al. 2007) and has been found to be attractive to honey bees and bumblebees (Loper et al. 1974, Pecetti et al. 2002, Mena Granero et al. 2005). Early flowering species shared pollinators, which for the most part were generalists (Bosch et al. 2009). Non-dominant species would benefit from an increased capacity to attract pollinators and thus compensate for their low abundance. The existence of this shared long-range attraction odor does not prevent the existence of short-range differences among species that may lead to pollinator specialization. At least this seems to be the case in the genus *Ranunculus*, where it was found that β-ocimene presents an interesting spatial emission pattern within the flower with a marked increase in the emissions from the apical to basal part of the petals (nectariferous) paralleling optical nectar-guide patterns, and emission of protoanemonin associated exclusively with pollen and reproductive parts of the flowers (Bergström et al. 1995, Jürgens & Dötterl 2004). While floral odours would operate at longer distances, the distinctiveness of the pollen's volatile profile suggests that it may serve a signaling role for pollinators specialized in collecting its pollen.

Variation in floral scent emission throughout the season could be the result of phenotypic plasticity (Majetic et al. 2009b). The observed pattern could be attributed to a physiological flower response to pollinator abundance or to seasonal environmental changes (e.g., temperature, precipitation or air humidity). However, contrary to the expectations of a typical phenotypically plastic response, no significant seasonal trend was found in the studied species. Moreover, if anything, emissions would be expected to increase late in the season, when temperatures are higher and precipitation and air humidity lower

(Jakobsen & Olsen 1994). The observed patterns could also be due to phylogenetic constraints, but there was no significant phylogenetic signal for the total terpene emissions and the phylogenetic signal for β-ocimene percentage was not sufficient to explain the differences between the less abundant species on the one hand and *R. officinalis* and *T. vulgaris* on the other. Alternatively, the observed inter-specific differences in scent signals may be the result of adaptive processes. Parachnowitsch et al. (2012) found floral scent to be under stronger natural selection than either flower size or color, which are much more frequently examined in studies of floral evolution. Successful pollinator attraction and ultimate sexual reproduction in a plant species depend not only on the efficiency of its own scent signal but also on the efficiency of the signals of co-flowering species, in combination with their relative abundances, distribution and spatial intermixing. Thus, scent emission is likely to be under strong selective pressure conditioned by seasonal pollinator availability, and plant community species composition. The seasonal pattern could result from selection for high flower attractiveness under low pollinator availability. The scent pattern found in species co-flowering with the dominant *R. officinalis* and *T. vulgaris* may result from selection of those species with a scent detectable for pollinators even in the presence of the abundant scent of the dominant species.

With few exceptions (Junker et al. 2010), studies analyzing the factors underlying the structure of plant-pollinator networks have mostly focused on abundance (neutrality models) and complementary phenological and morphological traits (trait matching models), while the potential contribution of volatiles has been largely ignored (Raguso 2008). For the first time we show a clear divergent seasonal pattern of scent emission in a plant-pollinator community, with different levels of investment in scent advertisement, and unveil contrasting plant-signaling strategies associated with pollinator seasonal abundance and local plant abundance. Overall, we provide a first community-wide description of the seasonal dynamics of flower scent emissions, and report patterns that suggest a key role of flower scent signals in structuring plant-pollinator networks.

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Appendix

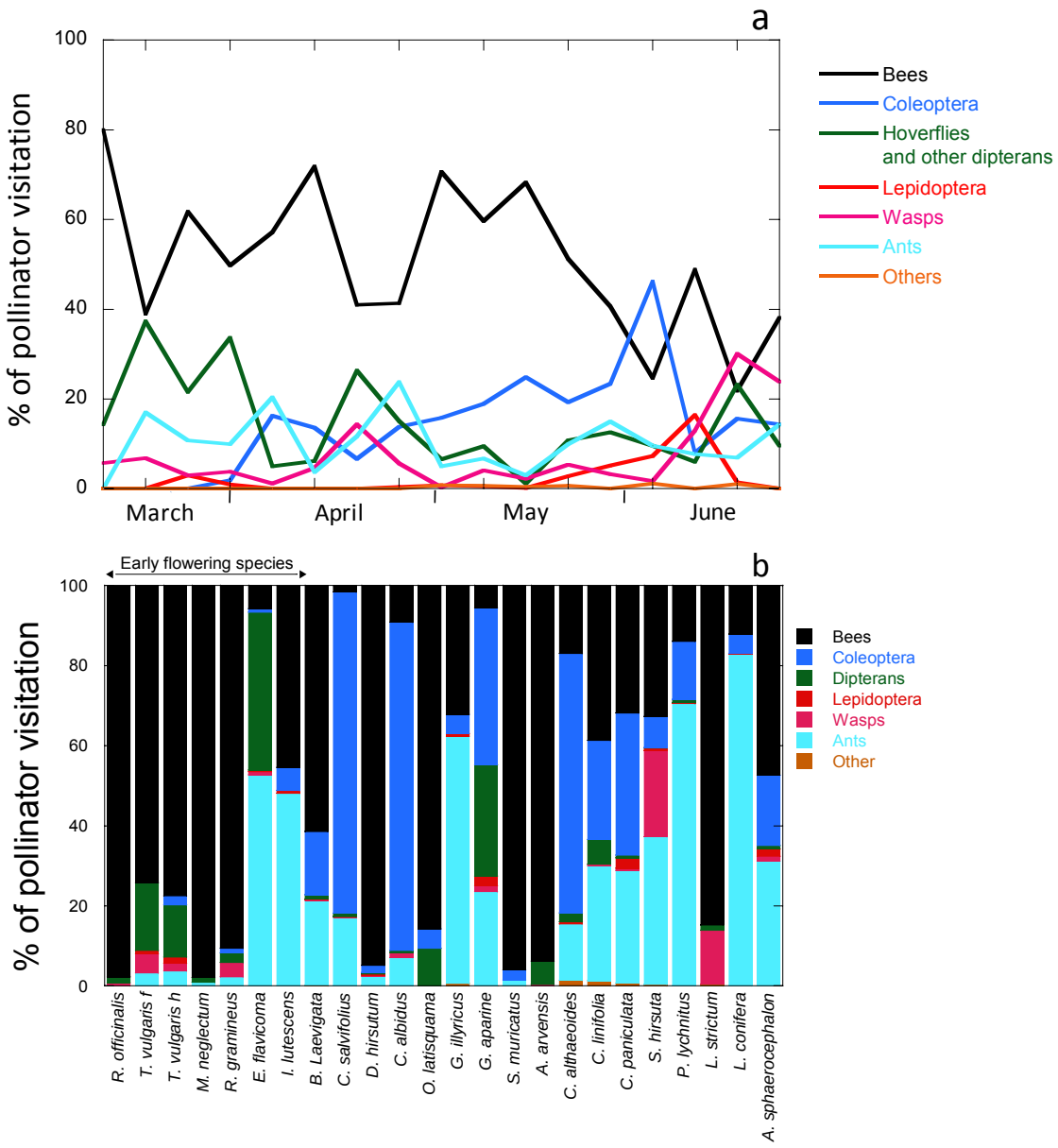

Fig. S1. a. Distribution of pollinator visitation by pollinator group throughout the season. b. Percentage of visitation rates of each pollinator group to each plant species of the Garraf shrubland community ordered by date of flowering peak.

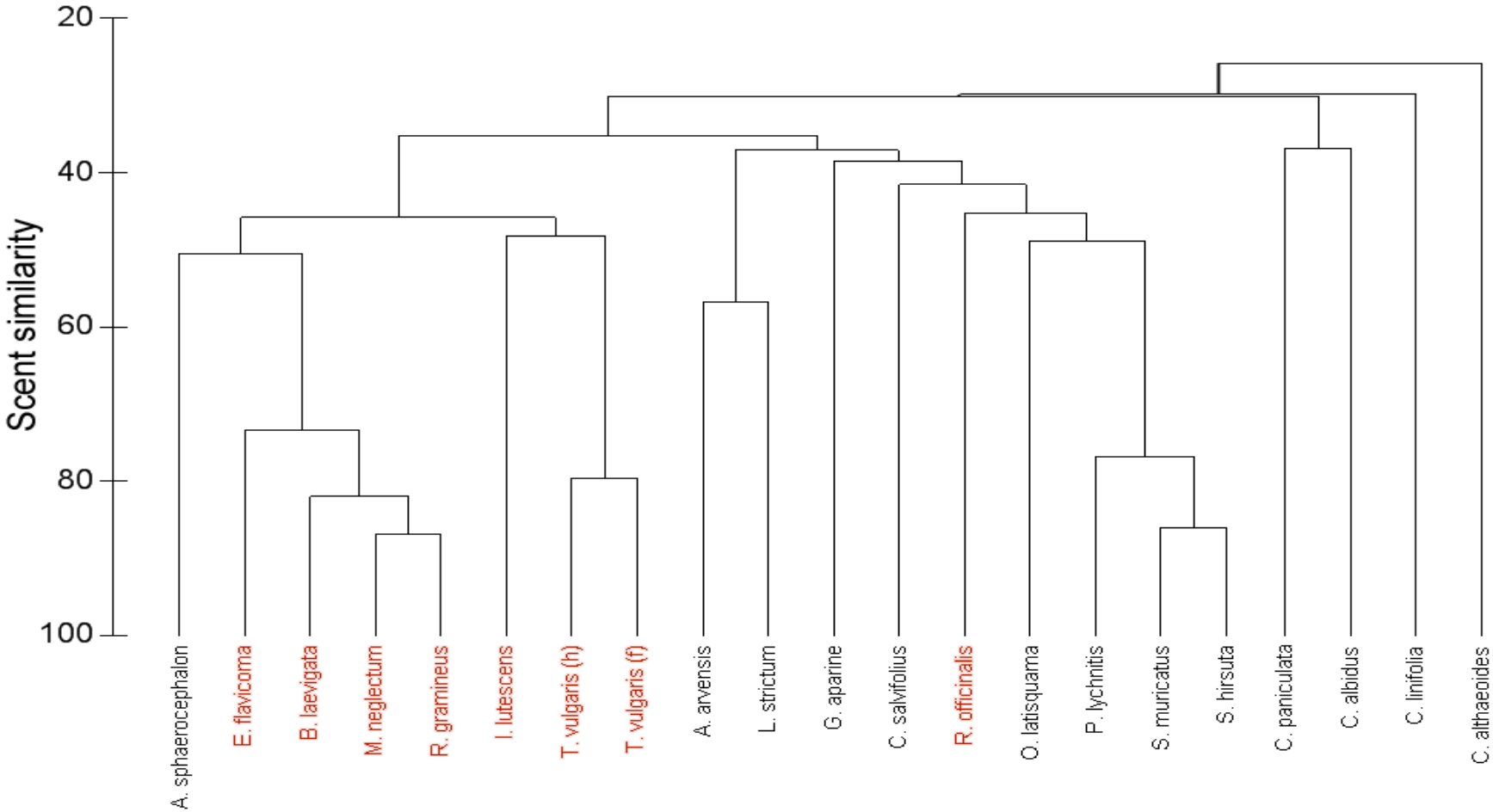

Fig. S2. Cluster analysis of the percentage of the different biogenic volatile organic compounds emitted by the plant species of the Garraf shrubland community. Species in red are early flowering species. Species in black are late flowering species.

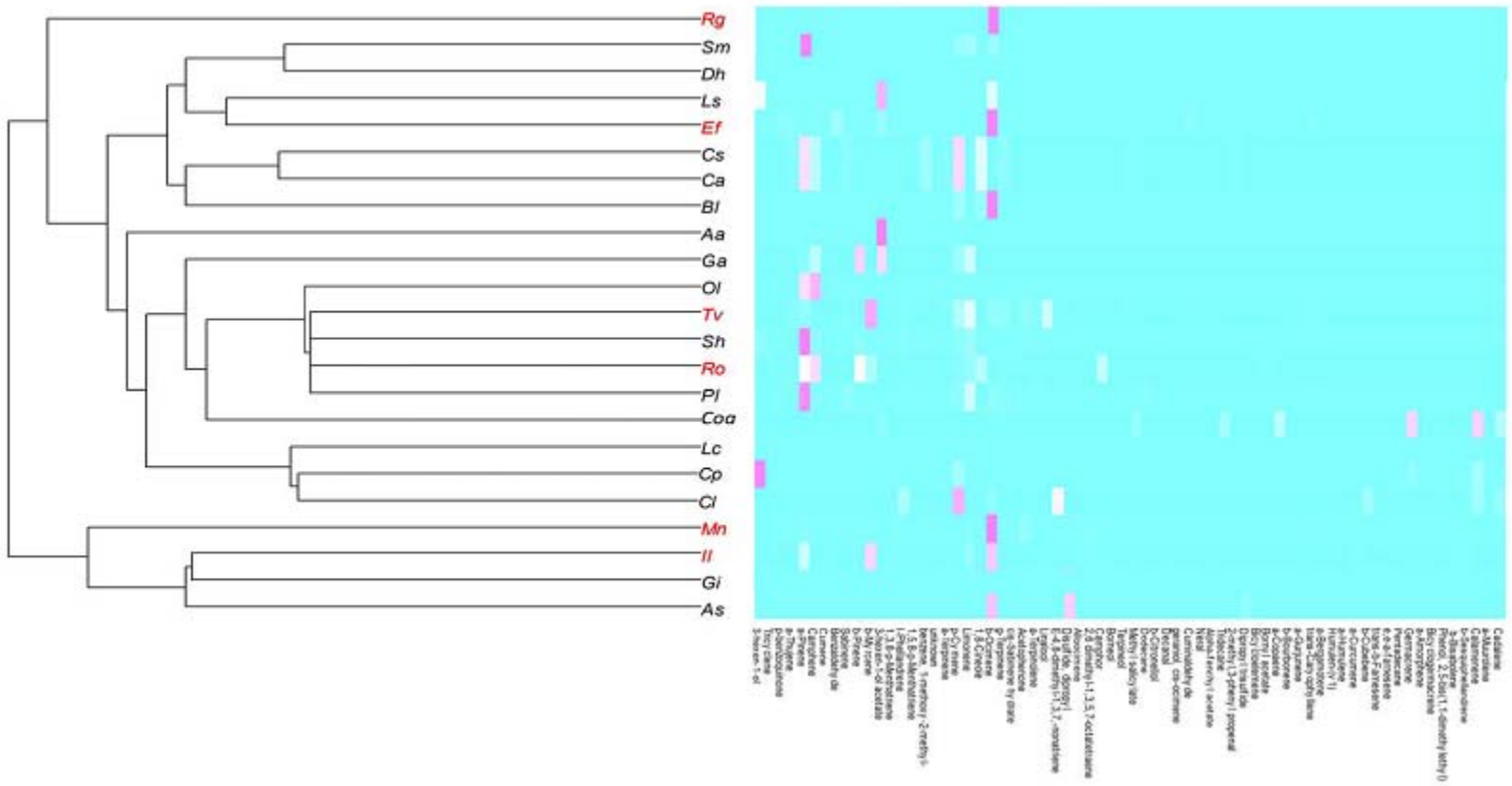

Fig. S3 Phylogenetic tree of the studied species and heatmap of the percentage of each emitted compounds in each species. Species abbreviations as in Fig. 2. Species in red in the phylogenetic tree are early flowering species. Species in black are late flowering species. The percentage range from low (blue) to high (purple) values (see Table S1-part 1 and 2- for the exact percentages).

	Ro	*Tvh*	*Tvf*	*Mn*	*Rg*	*Ef*	*Il*	*Bl*	*Cs*	*Ca*	*Ol*
3-Hexen-1-ol	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Tricyclene*	0,8 ± 0,25	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
p-Benzoquinone	- ± -	- ± -	- ± -	- ± -	- ± -	1,97 ± 1,18	- ± -	- ± -	- ± -	- ± -	- ± -
a-Thujene*	0,64 ± 0,2	1,48 ± 0,55	0,94 ± 0,64	0,03 ± 0,02	- ± -	1,95 ± 1,25	- ± -	- ± -	1,77 ± 0,19	- ± -	- ± -
a-Pinene*	19,7 ± 1,86	3,35 ± 0,79	1,12 ± 0,58	3,19 ± 1,52	- ± -	- ± -	16,19 ± 4,98	- ± -	27,77 ± 2,33	13,4 ± 13,4	43,76 ± 5,9
Camphene*	25,48 ± 2,55	0,62 ± 0,13	1,15 ± 0,63	- ± -	- ± -	- ± -	- ± -	- ± -	10,36 ± 2,12	- ± -	56,24 ± 5,9
Cumene*	- ± -	- ± -	0,43 ± 0,26	0,31 ± 0,17	0,48 ± 0,2	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Benzaldehyde	- ± -	- ± -	- ± -	0,03 ± 0,01	- ± -	8,65 ± 3,9	0,03 ± 0,03	- ± -	- ± -	- ± -	- ± -
Sabinene*	- ± -	0,8 ± 0,33	0,34 ± 0,34	0,09 ± 0,05	- ± -	- ± -	- ± -	- ± -	1,98 ± 0,31	- ± -	- ± -
b-Pinene*	18,98 ± 1,41	0,86 ± 0,14	0,7 ± 0,23	0,16 ± 0,07	- ± -	- ± -	0,21 ± 0,08	- ± -	- ± -	- ± -	- ± -
b-Myrcene*	7,42 ± 2,65	36,69 ± 4,7	29,71 ± 1,43	0,42 ± 0,19	- ± -	- ± -	36,26 ± 15,8	- ± -	- ± -	- ± -	- ± -
3-Hexen-1-ol acetate	- ± -	- ± -	- ± -	- ± -	- ± -	6,74 ± 3,6	- ± -	- ± -	- ± -	- ± -	- ± -
1,3,8-p-Menthatriene*	- ± -	- ± -	- ± -	0,39 ± 0,14	0,41 ± 0,02	- ± -	0,48 ± 0,27	- ± -	- ± -	- ± -	- ± -
I-Phellandrene*	- ± -	1,04 ± 0,2	1,02 ± 0,25	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
1,5,8-p-Menthatriene*	- ± -	- ± -	- ± -	0,04 ± 0,06	0,15 ± 0,06	- ± -	0,07 ± 0,07	- ± -	- ± -	- ± -	- ± -
Benzene, 1-methoxy-2-methyl-	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	5,51 ± 1,82	- ± -	- ± -
Unknown	- ± -	- ± -	- ± -	- ± -	0,36 ± 0,02	0,1 ± 0,1	- ± -	- ± -	- ± -	- ± -	- ± -
a-Terpinene*	- ± -	0,5 ± 0,19	0,49 ± 0,11	- ± -	- ± -	- ± -	- ± -	- ± -	1,56 ± 0,29	- ± -	- ± -
p-Cymene*	1,41 ± 0,43	8,51 ± 3,71	6,47 ± 3,1	0,22 ± 0,05	0,15 ± 0,07	0,18 ± 0,12	0,25 ± 0,08	10,05 ± 2,16	29,21 ± 3,51	16,3 ± 3,4	- ± -
Limonene*	3,74 ± 1,13	17,03 ± 3,37	15,01 ± 2,12	0,65 ± 0,43	- ± -	- ± -	4,18 ± 0,42	- ± -	- ± -	6,5 ± 1,4	- ± -
1,8-Cineole*	8,65 ± 1,55	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	8,51 ± 7,89	16,73 ± 4,59	- ± -	- ± -
b-Ocimene*	- ± -	3,4 ± 1,38	5,46 ± 1,64	84,46 ± 1,96	96,41 ± 1,02	70,46 ± 10,76	38,6 ± 13,39	81,44 ± 18,62	- ± -	- ± -	- ± -
g-Terpinene*	0,64 ± 0,22	3,56 ± 1,2	2,92 ± 1,49	0,08 ± 0,04	- ± -	- ± -	- ± -	- ± -	2,61 ± 0,79	- ± -	- ± -
cis-Sabinene hydrate*	- ± -	- ± -	1,99 ± 1,73	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Acetophenone	- ± -	3,69 ± 1,53	- ± -	5,36 ± 0,69	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
a-Terpinolene*	0,28 ± 0,03	1,4 ± 0,58	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -

	Ro	*Tvh*	*Tvf*	*Mn*	*Rg*	*Ef*	*Il*	*Bl*	*Cs*	*Ca*	*Ol*
Linalool*	- ± -	13,96 ± 7,48	19,72 ± 6,65	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
E-4,8-Dimethyl-1,3,7,-nonatriene*	- ± -	0,28 ± 0,1	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Disulfide, dipropyl	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Alloocimene*	- ± -	0,43 ± 0,23	0,77 ± 0,35	0,7 ± 0,13	0,29 ± 0,17	- ± -	1,13 ± 0,37	- ± -	- ± -	- ± -	- ± -
2,6 Dimethyl-1,3,5,7-octatetraene*	- ± -	- ± -	- ± -	0,86 ± 0,22	0,8 ± 0,1	- ± -	0,95 ± 0,31	- ± -	- ± -	- ± -	- ± -
Camphor*	10,5 ± 2,64	0,18 ± 0,08	0,37 ± 0,22	0,03 ± 0,02	- ± -	- ± -	0,11 ± 0,07	- ± -	- ± -	- ± -	- ± -
Borneol*	0,62 ± 0,11	- ± -	0,3 ± 0,25	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Terpineol*	- ± -	0,43 ± 0,13	0,42 ± 0,17	- ± -	- ± -	- ± -	- ± -	- ± -	0,69 ± 0,24	- ± -	- ± -
Methyl salicylate	- ± -	- ± -	- ± -	0,1 ± 0,06	- ± -	- ± -	- ± -	- ± -	0,69 ± 0,11	- ± -	- ± -
Dodecane*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
b-Citronellol*	- ± -	- ± -	0,28 ± 0,25	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Decanal	0,18 ± 0,02	- ± -	- ± -	0,04 ± 0,02	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Geraniol, cis-ocimene*	- ± -	- ± -	2,9 ± 2,47	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Cuminaldehyde	- ± -	0,34 ± 0,05	- ± -	- ± -	0,02 ± 0,01	2,65 ± 1,37	- ± -	- ± -	- ± -	- ± -	- ± -
Neral*	- ± -	0,05 ± 0,03	1,15 ± 1,02	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
a-Fenchyl acetate	- ± -	0,09 ± 0,03	0,43 ± 0,18	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Tridecane	- ± -	- ± -	- ± -	0,05 ± 0,03	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
2-Methyl,3-phenyl propenal	- ± -	0,1 ± 0,01	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,79 ± 0,27	- ± -	- ± -
Dipropyl trisulfide	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Bicycloelemene*	- ± -	- ± -	0,17 ± 0,03	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Bornyl acetate*	0,66 ± 0,3	- ± -	- ± -	- ± -	- ± -	1,23 ± 0,85	- ± -	- ± -	- ± -	- ± -	- ± -
a-Copaene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
b-Bourbonene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	9,53 ± 1,32	- ± -
a-Gurjunene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	6,49 ± 3,26	- ± -
trans-Caryophyllene*	0,02 ± 0,02	1,48 ± 0,5	3,28 ± 1,01	1,57 ± 0,52	- ± -	2,69 ± 1,31	- ± -	- ± -	- ± -	5,29 ± 2,21	- ± -
a-Bergamotene*	- ± -	- ± -	0,22 ± 0,04	0,37 ± 0,19	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -

	Ro	*Tvh*	*Tvf*	*Mn*	*Rg*	*Ef*	*Il*	*Bl*	*Cs*	*Ca*	*Ol*
a-Humulene*	- ± -	- ± -	0,23 ± 0,08	0,15 ± 0,04	- ± -	1,01 ± 0,65	- ± -	- ± -	- ± -	1,20 ± 0,49	- ± -
a-Curcumene*	- ± -	- ± -	- ± -	0,17 ± 0,07	- ± -	- ± -	0,7 ± 0,21	- ± -	- ± -	13,10 ± 6,29	- ± -
b-Cubebene*	- ± -	0,01 ± 0,01	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
trans-b-Farnesene*	- ± -	- ± -	0,18 ± 0,05	0,09 ± 0,08	- ± -	- ± -	0,25 ± 0,08	- ± -	- ± -	- ± -	- ± -
e,e-a-Farnesene*	- ± -	- ± -	- ± -	0,22 ± 0,15	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Pentadecane	- ± -	- ± -	- ± -	- ± -	0,09 ± 0,06	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Germacrene*	- ± -	- ± -	0,18 ± 0,02	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	1,18 ± 0,53	- ± -
a-Amorphene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	7,67 ± 2,20	- ± -
Bicyclogermacrene*	- ± -	0,16 ± 0,05	0,71 ± 0,18	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Phenol, 2,5-bis(1,1-dimethylethyl)	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,34 ± 0,12	3,58 ± 0,83	- ± -
b-Bisabolene*	- ± -	- ± -	- ± -	0,15 ± 0,08	- ± -	- ± -	0,13 ± 0,04	- ± -	- ± -	- ± -	- ± -
b-Sesquiphellandrene*	- ± -	- ± -	- ± -	0,01 ± 0,02	- ± -	- ± -	0,21 ± 0,06	- ± -	- ± -	- ± -	- ± -
Calamenene*	- ± -	0,04 ± 0,02	0,22 ± 0,05	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	10,99 ± 2,39	- ± -
a-Muurolene*	- ± -	- ± -	0,04 ± 0,02	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Cadalene*	- ± -	- ± -	0,09 ± 0,01	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	4,75 ± 0,95	- ± -

Table S1 (part 1) BVOC's detected in GC analysis for the following plants of the community: *Rosmarinus officinalis* (Ro); *Thymus vulgaris* h (Tvh*); Thymus vulgaris* f (Tvf); *Muscari neglectum* (Mn); *Ranunculus gramineus* (Rg); *Euphorbia flavicoma* (Ef); *Iris lutescens* (Il); *Biscutella laevigata* (Bl); *Cistus salvifolius* (Cs); *Cistus albidus* (Ca); *Orobanche latisquama* (Ol). *Dorycnium hirsutum*, *Leuzea conifera* and *Gladiolus illyricus* are not shown in the table since no significant amounts of any BVOC was detected in our GC analysis.

	Ga	*Sm*	*Aa*	*Coa*	*Cl*	*Cp*	*Sh*	*Pl*	*Ls*	*As*
3-Hexen-1-ol	- ± -	- ± -	- ± -	- ± -	- ± -	73,67 ± 6,78	3,8 ± 1,57	- ± -	28,5 ± 15,62	- ± -
Tricyclene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
p-Benzoquinone	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
a-Thujene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,41 ± 0,08	1,12 ± 0,31	- ± -	- ± -
a-Pinene*	2,28 ± 0,88	76,37 ± 0,58	- ± -	- ± -	- ± -	- ± -	74,39 ± 2,7	60,76 ± 9,17	- ± -	- ± -
Camphene*	12,06 ± 2,89	- ± -	- ± -	- ± -	- ± -	- ± -	4,03 ± 0,39	1,38 ± 0,85	- ± -	- ± -
Cumene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Benzaldehyde	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Sabinene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,6 ± 0,07	3,21 ± 1,74	- ± -	- ± -
b-Pinene*	33,32 ± 7,05	- ± -	- ± -	- ± -	- ± -	- ± -	3,17 ± 0,31	- ± -	- ± -	- ± -
b-Myrcene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
3-Hexen-1-ol acetate	31,75 ± 14	- ± -	100 ± 0,0	2,6 ± 2,47	- ± -	- ± -	- ± -	- ± -	48,56 ± 9,85	- ± -
1,3,8-p-Menthatriene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
I-Phellandrene*	- ± -	- ± -	- ± -	- ± -	8,96 ± 2,59	- ± -	1,66 ± 0,92	0,96 ± 0,23	- ± -	- ± -
1,5,8-p-Menthatriene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Benzene, 1-methoxy-2-methyl-	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Unknown	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
a-Terpinene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,12 ± 0,02	0,27 ± 0,2	- ± -	- ± -
p-Cymene*	5,56 ± 1,51	7,28 ± 1,11	- ± -	- ± -	41,79 ± 4,66	11,02 ± 1,53	2,66 ± 1,01	3,85 ± 1,59	- ± -	- ± -
Limonene*	15,03 ± 2,94	8,72 ± 1,04	- ± -	- ± -	- ± -	- ± -	7,16 ± 1,39	20,19 ± 5,84	- ± -	- ± -
1,8-Cineole*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
b-Ocimene*	- ± -	7,63 ± 1,6	- ± -	- ± -	4,21 ± 1,93	- ± -	- ± -	3,19 ± 1,98	22,94 ± 9,9	49,78 ± 6,08
g-Terpinene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,45 ± 0,14	2,34 ± 0,96	- ± -	- ± -
cis-Sabinene hydrate*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Acetophenone	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
a-Terpinolene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	1,12 ± 0,07	0,56 ± 0,37	- ± -	- ± -

	Ga	*Sm*	*Aa*	*Coa*	*Cl*	*Cp*	*Sh*	*Pl*	*Ls*	*As*
Linalool*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
E-4,8-Dimethyl-1,3,7,-nonatriene*	- ± -	- ± -	- ± -	- ± -	27,44 ± 6,46	- ± -	- ± -	- ± -	- ± -	- ± -
Disulfide, dipropyl	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	45,82 ± 5,62
Alloocimene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
2,6 Dimethyl-1,3,5,7-octatetraene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Camphor*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Borneol*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Terpineol*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Methyl salicylate	- ± -	- ± -	- ± -	3,62 ± 1,74	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Dodecane*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	1,37 ± 0,89	- ± -	- ± -
b-Citronellol*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Decanal	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Geraniol, cis-ocimene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Cuminaldehyde	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,07 ± 0,01	0,16 ± 0,15	- ± -	- ± -
Neral*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
a-Fenchyl acetate	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Tridecane	- ± -	- ± -	- ± -	6,29 ± 3,21	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
2-Methyl,3-phenyl propenal	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Dipropyl trisulfide	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	4,4 ± 0,77
Bicycloelemene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Bornyl acetate*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
a-Copaene*	- ± -	- ± -	- ± -	10,56 ± 5,03	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
b-Bourbonene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
a-Gurjunene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
trans-Caryophyllene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,37 ± 0,21	- ± -	- ± -
a-Bergamotene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Humulen-(v1)*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -

	Ga	*Sm*	*Aa*	*Coa*	*Cl*	*Cp*	*Sh*	*Pl*	*Ls*	*As*
a-Humulene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,16 ± 0,16	- ± -	- ± -
a-Curcumene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
b-Cubebene*	- ± -	- ± -	- ± -	- ± -	6,05 ± 3,22	- ± -	- ± -	- ± -	- ± -	- ± -
trans-b-Farnesene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
e,e-a-Farnesene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Pentadecane	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Germacrene*	- ± -	- ± -	- ± -	29,93 ± 13,74	- ± -	4,31 ± 1,48	- ± -	- ± -	- ± -	- ± -
a-Amorphene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Bicyclogermacrene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Phenol, 2,5-bis(1,1-dimethylethyl)	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	0,01 ± -	- ± -	- ± -	- ± -
b-Bisabolene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
b-Sesquiphellandrene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Calamenene*	- ± -	- ± -	- ± -	30,42 ± 11,35	6,53 ± 3,65	9,73 ± 3,41	0,04 ± 0,01	- ± -	- ± -	- ± -
a-Muurolene*	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -	- ± -
Cadalene*	- ± -	- ± -	- ± -	9,2 ± 4,4	5,02 ± 1,55	1,28 ± 1,28	- ± -	- ± -	- ± -	- ± -

Table S1 (part 2) BVOC's detected in GC analysis for the following plants of the community: *Gallium aparine* (Ga); *Scorpiurus muricatus* (Sm*); Anagallis arvensis* (Aa); *Convolvulus althaeoides* (Coa); *Centaurea linifolia* (Cl); *Centaurea paniculata* (Cp); *Siderites hirsuta* (Sh); *Phlomis lychnitis* (Pl); *Linum strictum* (Ls); *Allium sphaerocephalon* (As). *Dorycnium hirsutum*, *Leuzea conifera* and *Gladiolus illyricus* are not shown in the table since no significant amounts of any BVOC was detected in our GC analysis.

Chapter 3

A syrphid fly uses olfactory cues to find a non-yellow flower

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A syrphid fly uses olfactory cues to find a non-yellow flower

Abstract

Syrphid flies are frequent flower visitors, but little is known about the cues they use to find flowers. We determined the importance of visual and olfactory cues in a flight cage bioassay using *Cirsium arvense* (Asteraceae) flower heads and experienced *Episyrphus balteatus* (Diptera, Syrphidae). We tested the response of antennae of the flies to headspace inflorescence scent samples by using gas chromatography coupled to electroantennography (GC-EAD). The bioassay revealed that both sexes of experienced flies rely on olfactory, not visual, cues to find *C. arvense* flower heads. The GC-EAD measurements demonstrated that male and female flies have olfactory receptors for several of the compounds emitted by the inflorescences. These electroantennographic-active compounds may be responsible for the attraction of flies to the *C. arvense* flower heads. Among the compounds eliciting an antennal response are methyl salicylate and 2-phenylethanol, which were previously described as syrphid attractants. Overall, our study demonstrates for the first time that a syrphid fly uses olfactory and not visual cues to find a pollen/nectar host-plant.

3.1. Introduction

Syrphids (Diptera, Syrphidae) frequently visit flowers to feed on their pollen and nectar (Shi et al. 2009). It has been assumed that these flies primarily use vision to seek floral feeding sites, and flower colour, size, and shape preferences of syrphids have been studied (e.g., Lunau and Wacht 1994, Sutherland et al. 1999, Lunau et al. 2005, Shi et al. 2009). Among the visual floral cues, colour seems to be the most important, and syrphids repeatedly have been found to respond to yellow, and to prefer yellow over other colours (Lunau 1988, Sutherland et al. 1999). This colour preference may help the flies find pollen that often is yellow (Lunau and Wacht 1994).

Besides visual plant cues, olfactory cues also may play a role in hoverfly attraction. Volatile organic compounds increase catches of hoverflies in yellow sticky traps (Zhu and Park 2005) and elicit searching behaviour for ovoposition sites (Harmel et al. 2007). However, almost nothing is known about the importance of olfactory compared to visual cues for finding nectar and pollen (Majetic et al. 2009).

Although yellow is highly attractive for syrphids, they also visit other coloured flowers. The floral cues for finding non-yellow flowers are unknown. *Cirsium arvense* (L.) Scop. (Asteraceae), our study plant, has pink flower heads that are visited by a large variety of insects, among them syrphids (Theis et al. 2007). *Episyrphus balteatus* De Geer is the most abundant syrphid visitor (Primante and Dötterl unpublished data). In the present work, we focused on the relative importance of visual and olfactory cues of *C. arvense* flower heads for host-plant identification by experienced *E. balteatus*. Additionally, we tested headspace inflorescence scent samples on the antennae of flies by gas chromatography coupled to electroantennography (GC-EAD) in order to identify which compounds emitted by *C. arvense* inflorescence are perceived by the fly.

3.2. Materials and Methods

3.2.1. Plant material and volatile collection

The *Cirsium arvense* plants were from the Ecological Botanical Garden of the University of Bayreuth. *C. arvense* is a dioecious plant. Staminate and pistillate flower heads emit the same scent compounds, although the total amount of scent is higher in staminate flower heads (Theis et al. 2007, Primante and Dötterl unpublished data). The petals of both sexes reflect light mainly in the blue (max. 450 nm) and red range (650-700 nm) (Primante and Dötterl unpublished data; Fig. 1). We, therefore, did not discriminate between flower sexes.

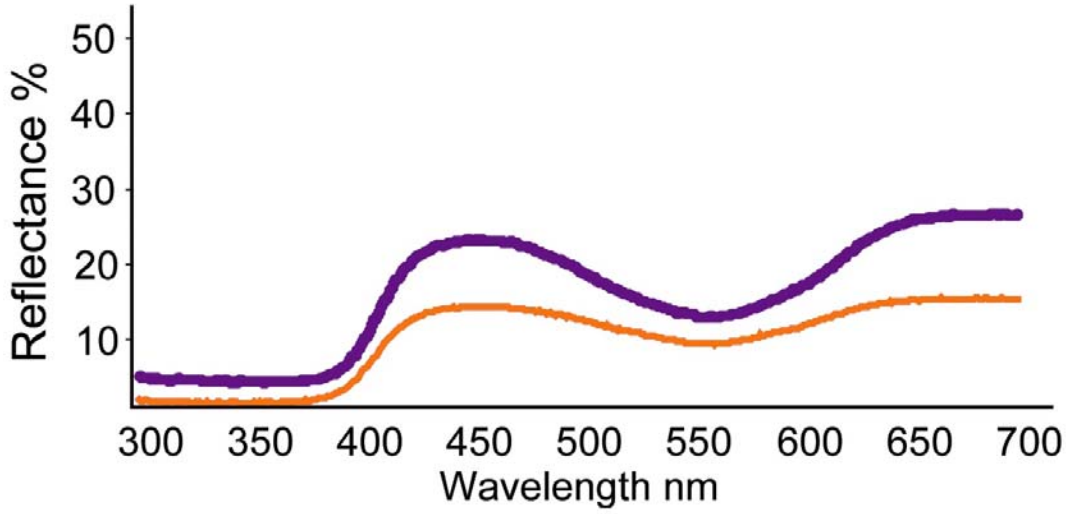

Fig. 1 Light reflectance of staminate and pistillate flower heads of *Cirsium arvense*. Petals of both sexes reflect light mainly in the blue (max. 450 nm) and the red range (650-700 nm).

Flowering branches were cut in the field and placed in water in the laboratory for immediate scent collection. Four to seven flower heads of *C. arvense* were enclosed in a polyester oven bag (Toppits, Germany), and over an 8 h period the emitted volatiles were trapped in an adsorbent tube filled with 20 mg of a 1:1 mixture of Tenax-TA (mesh 20-40, Supelco) (Dötterl et al. 2005). Volatiles were eluted with 60 µl of acetone (SupraSolv, Merck KgaA, Germany) to obtain 6 odour samples for the electrophysiological experiments.

3.2.2. Electrophysiological experiments and chemical analyses

Electrophysiological analyses of the floral scent samples were performed with the GC-EAD system as described by Dötterl et al. (2005). Antennae from 23 females and 11 males of *E. balteatus* (one antenna per individual, one run per antenna) were tested between July and August 2008 on our 6 odour samples (3-5 female and 1-5 male antennae per sample). To identify the EAD-active compounds, 1 µl of the scent samples was analyzed on a Varian 3800 gas chromatograph fitted with a 1079 injector and a ZB-5 column (5% phenyl polysiloxane; length, 60 m; inner diam, 0.25 mm; film thickness, 0.25 µm; Phenomenex) and a Varian Saturn 2000 mass spectrometer (Dötterl et al. 2005). Component identification was

carried out using the NIST 08 mass spectral database or MassFinder 3, and was confirmed by comparison of mass spectra and retention times with those of authentic standards.

3.2.3. Behavioural assay

To determine the relative importance of olfactory and visual cues for finding flowering *C. arvense* by flies experienced in foraging, we conducted two-choice bioassays in a flight cage at the end of summer 2009.

Pupae of *E. balteatus* were provided by Katz Biotech Ag (Baruth, Germany) and kept in a small gauze tent (60x60x60 cm) at 23ºC until hatching. Immediately after the adults hatched, they all were transferred to a flight cage (7.20x3.60x2.20 m) that was set up in a greenhouse. The population of 55 female and 30 male adult flies was fed on fresh flowers of *C. arvense*, but all floral material was removed from the cage at least 12 h before conducting a bioassay.

We performed two two-choice bioassays to assess fly attraction to floral cues: 1) Visual only vs. Visual and olfactory cues combined and 2) Olfactory only vs. Visual and olfactory cues combined. In the assays, flies were offered flowering branches (with 30-40 flower heads each) in quartz glass cylinders constructed to present either visual and olfactory cues, or both. The basic cylinder for testing attraction to olfactory and visual cues in combination consisted of a transparent quartz glass cap and body and a sleeve of Macrolon®, which connected nad sealed the cap and the body. The Macrolon® sleeve had 60 holes (diam 0.2 cm), arranged in three horizontal lines to allow diffusion of floral scents. The cylinders were mounted on a black polyvinyl chloride (PVC) disc (diam 11 cm) that was attached to a square wooden table. A tube coupled the cylinder to a membrane pump (flow 1 lmin ^{1}; G12/01EB, Rietschle Thomas, Puchheim, Germany). A modified transparent cylinder without holes and without connection to a pump was used for testing visual attraction only. A cylinder with holes and the pump, but painted black with semi-matte varnish, was used for testing olfactory attraction only (Fig. 2)

Fig. 2 Flower heads of *Cirsium arvense* placed in quartz glass cylinders employed for behavioral assays. Cylinders were constructed to release either visual (V) or olfactory cues (O) only or both (V+O).

The two cylinders were set up 2 m apart for each of the two bioassays. Each test was conducted for 40 min, and 20 min after beginning the test, the position of the cylinders was exchanged. The behaviour of the flies was classified as "approaching" when flies hovered in front of the cylinder but did not land and "landing" when flies contacted the glass cylinder. To assure that an individual fly was counted only once in a specific test, responding flies were captured.

3.3. Results and Discussion

In the bioassays, *E. balteatus* preferred a combination of both cues modalities over visual cues but not over olfactory cues. When testing a combination of visual and olfactory cues against visual cues, 24 flies were attracted by the combination of both cues and no flies were attracted by the visual cues (*chi square* observed vs. expected test: χ^2= 24.0; *df*: 1; P<0.001) (Table 1). When testing a combination of visual and olfactory cues against olfactory cues, both types of

cylinders had the same attractiveness (*chi square* observed vs. expected test: χ^2= 0,36; *df*: 1; P= 0.55) (Table 1).

	VISUAL		vs.	**VISUAL+OLFACTORY**	
Test 1	Landing	Approaching		Landing	Approaching
	0	0		18(11♀; 7♂)	6(5♀; 1♂)
	OLFACTORY		vs.	**VISUAL+OLFACTORY**	
Test 2	Landing	Approaching		Landing	Approaching
	7(3♀; 4♂)	4(4♀)		10(8♀; 1♂)*	4(3♀)*

Table 1 Attractiveness of combined visual and olfactory cues in comparison to decoupled visual (Test 1) and olfactory (Test 2) cues for experienced female and male *Episyrphus balteatus*. *The sex of one fly was not determined.

These experiments demonstrate that experienced flies primarily use olfactory cues for seeking *C. arvense* flower heads, whereas visual cues do not play a significant role. We did not determine whether flies would respond to visual cues in the absence of *C. arvense* odours. *C. arvense* flowers reflect light in the blue range of light and blue was somewhat attractive to *E. balteatus* (Sutherland et al. 1999).

The GC-EAD measurements revealed the candidate molecules responsible for attraction of flies. Four compounds occurring in the scent of *C. arvense* flower heads (Theis et al. 2007), consistently elicited antennal responses, in more that 50% of tested antennae from both female and male flies: phenylacetaldehyde, methyl salicylate, dimethyl salicylate, and pyranoid linalool oxide (Fig. 3). Less consistent responses were found for the two coeluting compounds methyl benzoate, which was not described by Theis et al. (2007), and linalool (47% response rate); and for 2-phenylethanol (41% response rate). Although we did not test the isolated EAD-active compounds for attractiveness, our results, and those of other researchers, led us to hypothesize that at least methyl salicylate and 2-phenylethanol are attractants for *E. balteatus*. These compounds were emitted in abundant amounts from the flower heads used in this study (Fig. 3), and have been described previously as syrphid attractants (Zhu and Park 2005).

In a field experiment, they increased the attractiveness of yellow sticky cards (Zhu and Park 2005).

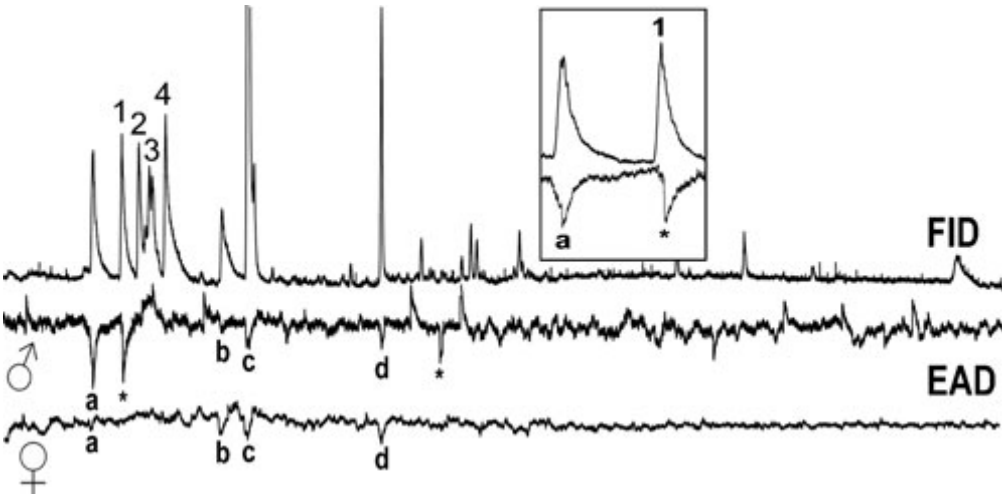

Fig. 3 Examples of coupled gas chromatographic and electroantennographic detection (GC-EAD) of a *Cirsium arvense* inflorescence scent sample using antennae of female and male Episyrphus balteatus. a: phenylacetaldehyde; b: (E+Z)-pyranoid linalool oxide; c: methyl salicylate; d: dimethyl salicylate. The abundant compounds acetophenone (1), (E)-furanoid linalool oxide (2), coeluting linalool and methyl benzoate (3) and 2-phenylethanol (4) did not elicit responses. The responses marked with an asterisk are artifacts as shown in the box where we present an enlarged section of the FID and corresponding antennal response of the male. The antennal response did not occur simultaneously with FID peak 1.

Recently, Majetic et al. (2009) found that augmentation of inflorescences of *Hesperis matronalis* with scent (collected at night from *H. matronalis*) increased visitation by syrphids, whereas the colour of this colour-polymorphic plant (white vs. purple) did not influence the visitation rate of flies. Their study also showed that syrphid flies respond to floral scent, but they performed the experiments with manipulated inflorescences. Our study demonstrates for the first time that a syrphid fly uses olfactory and not visual cues to find an unmanipulated pollen/nectar host-plant.

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Conclusions

Conclusions

The main conclusions that can be drawn from the studies presented in this thesis are the following:

1) An ecological trait (flower density) and a phenological trait (flowering time) are the main factors structuring plant-pollinator interactions in our community. The two plant species mostly responsible for the observed structure are *Rosmarinus officinalis* and *Thymus vulgaris,* which largely dominate the community in terms of flower production and bloom early in the season. Because these two characters, especially flower density, may vary widely depending on the ecological context, their effect on the structure of plant-pollinator interactions may potentiate differences between different ecological scenarios.

2) Although to a lesser extent than flower density and phenology, corolla restrictiveness also emerged as a floral trait structuring plant-pollinator interactions. In our community, plants with restrictive corollas were weakly associated with nectarivorous Hymenoptera with long proboscis (in absolute terms and in relation to their body size).

3) Floral rewards, pollen and nectar, did not emerge as important drivers of plant-pollinator interaction structure. However, we found a weak association of Coleoptera and female bees, the two groups more strongly dependent on pollen, with plants producing large amounts of pollen per flower.

4) These associations between floral and pollinator traits result in a certain structure, defined by three pollinator clusters. These clusters are

characterized not only by taxonomic status but also by different pollinator traits such as activity period, body size and proboscis length.

5) A first community wide description of flower emissions is provided in this thesis. Again, seasonality plays an essential role with a two contrasting scent profile scenarios early and late in the season. Species blooming early in the season have greater flower emissions than species blooming later. This temporal pattern may reflect a mechanism whereby species blooming early, at a time when pollinators are scarce for the high numbers of flower available, invest more in scent advertisement.

6) We also found differences among early-blooming plant species in scent profile depending on their flower abundance. Les abundant (in terms of flower density) plants produce larger amounts of β-ocimene, a general pollinator attractant. We hypothesize that high emissions of this specific compound may confer increased capacity to attract pollinators, thus compensating low flower abundance.

7) Overall, seasonality, a major trait of Mediterranean systems, emerges as an essential driver of plant-pollinator dynamics in our community. Both plant and pollinator traits, as well as pollinator composition, have a strong phonological component.

8) There has been a lot of controversy over the relative roles of flower scent and colour as pollinator attractants. Diptera, in particular, have been considered to respond mostly to visual cues. We demonstrate, for the system between *Cirsium arvensis* and the syrphid fly *E. balteatus*, that although both types of cues are complementary, floral scent plays a major role in pollinator attraction.