Biotic and abiotic factors that determine the emission of volatile organic compounds by flowers

PhD Thesis

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to be eligible for the Doctor degree

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Abstract

Flowering plants present a great array of traits that act through different sensory channels to communicate with pollinators. Apart from offering nutritional rewards and using visual stimuli, flowers emit volatile organic compounds (VOCs) to attract pollinators and stimulate reproductive outcrossing. Floral scents act as chemical cues that enhance flower location by pollinators. They also provide information about the plant species, flower state, and available floral rewards. Some floral volatiles can play roles other than attraction, such as defense against herbivores. This duality of roles of floral emissions converts floral scents into complex mixtures of compounds with multiple effects on different organisms. The complexity of understanding and characterising floral emissions increases when considering that they are variable in time and space. They show circadian and phenological patterns of change that usually occur very fast, due to the ephemeral nature of flowers. Different flower parts can also show different emission profiles depending on their function. To all these sources of variability we can add diverse biotic and abiotic environmental factors that modify floral VOC emissions in many different ways.

The main objective of this thesis was to shed light on which are the factors that determine floral volatile emissions, and how do they affect these emissions and their ecological functions. In the first chapter of this thesis we reviewed the current knowledge on floral VOC emissions. We identified the open questions that still needed to be addressed or investigated in more detail in the research field of floral VOC emissions.

Floral emissions are first determined by the array of compounds that the species are able to produce and their potential biosynthetic and emission capacities, which are strongly related to the species biology. We thus analyzed how different aspects of the biology of the species can determine the emission profiles of flowers. In the second chapter, we tested and demonstrated that flowering plants pollinated by insects usually present higher diversities of floral volatiles and emit higher amounts of them, than do plants pollinated by wind which do not need floral volatiles for attractive purposes. Our studies also highlighted the importance of flowering phenology in the evolution of flower scents. In the third chapter, we tested whether well-known seasonal patterns of decreasing competition occurring every year in a community among co-flowering plants for pollinators led to the selection of a pattern of decreasing emission of flower volatiles and decreasing production of floral rewards along the flowering period of each species. In this case, floral rewards showed a trend to decrease in species with long flowering periods, coinciding with decreasing competition, while floral emissions showed no phenological trends of change in any case. In the fourth chapter we observed that plants adapt their physiology to optimize their floral emissions under the climatic conditions of the flowering period, by showing that optimum temperatures for floral emissions are well correlated with mean temperature of the flowering season.

Floral VOC emissions of the species are affected by environmental factors at the individual (organism) or tissular level. Many different biotic and abiotic agents can affect floral emissions by different ways. There are diverse physiological states of the plant that can substantially modify the emission profiles and amounts of floral VOCs. Basically, we can consider several stress responses that are activated in response to biotic and abiotic factors, some of which are poorly known with respect to their effects on floral VOC emissions. It is of high interest to find responses to the question of how all these aspects that act on plant physiology at different levels can affect floral emissions, and if such changes affect and how, the plant-pollinator interactions that they mediate.

Regarding biotic agents, we addressed the effect of floral microbiota and herbivores on flower VOC emissions. Our experiments demonstrated that the suppression of floral microbiota radically reduced and modified the composition of floral terpene emissions of *Sambucus nigra* plants, but not the internal tissular content, suggesting that the floral microbiota plays a crucial role in the quantity and quality of floral VOC emissions. We additionally conducted a series of experiments with *Diplotaxis erucoides* plants submitted to leaf and flower herbivory by *Pieris brassicae* caterpillars to test the effect of these two kinds of herbivory on floral emissions. Our results showed immediate responses in the attacked flowers with increases in floral emission rates of few compounds with known defensive functions. Leaf herbivory caused no changes in the emissions of intact flowers, but the combination of leaf herbivory with flower herbivory showed a synergistic effect with enhanced defensive response.

The research on the potential changes that floral emissions could experience in response to diverse drivers of Global Change, such as the temperature increase or the increase in tropospheric ozone concentrations, are of critical interest because of the diverse effects that such changes can have on the interactions that floral VOCs mediate. We thus addressed the effect of these two abiotic agents on floral emissions. Our measurements confirmed that temperature has a main positive effect on floral volatile emissions. They also revealed that temperature increases as those predicted for the next century as a result of Global Warming can lead to significant total increases in floral VOC emissions and also to important changes in floral scent relative composition, depending on the species. Finally, we tested the effect of ambient ozone on emitted floral VOCs, by analysing the chemical composition of the floral blends exposed to different ozone concentrations along different distances through a system of reaction chambers and the responses of pollinators. We detected degradation of some compounds and the formation of other ones by oxidation of original floral volatiles with ozone. Our behavioural tests indicated that all the changes observed in floral chemical cues with increasing ozone concentration and increasing distance of exposure resulted in the loss of attraction effect on pollinators.

This thesis thus provides new insights on the factors that determine floral volatile emissions and their repercussions on plant-pollinator interactions and warrant deep consideration of both biotic and abiotic factors driving floral scent chemistry and floral scent ecology in a continuously changing environment.

GENERAL INTRODUCTION

General Introduction

Plant volatiles: properties and functions

During their life, plants are exposed to a variety of detrimental conditions that they cannot avoid due to their condition of sessile organisms. They have thus evolved a great diversity of chemical compounds to deal with detrimental environmental conditions and the related stresses, while preventing the attack by harmful organisms (Holopainen & Gershenzon, 2010). Most of these compounds that mostly develop defense and protective functions are secondary metabolites (Swain, 1977; Hartmann, 1996; Bennett & Wallsgrove, 2006). Among these secondary metabolites, some present high volatilities and are actively released by different plant organs with different biological purposes (Dudareva *et al.*, 2006). Plants emit a great amount and diversity of volatile organic compounds (VOCs), belonging to the groups of terpenoids (isoprenoids), benzenoids, fatty acid derivatives and amino acid derivatives (Dudareva *et al.*, 2004). These compounds are commonly classified under the name of BVOCs (biogenic volatile organic compounds). In general, plant VOCs have high vapour pressures, low molecular weights, and varying lipid and water solubilities (Copolovici & Niinemets, 2005). These variables define the volatility, solubility and the diffusion of the compounds among different cellular phases, which are the most relevant physical properties affecting plant VOC emissions (Niinemets *et al.*, 2004; Noe *et al.*, 2006).

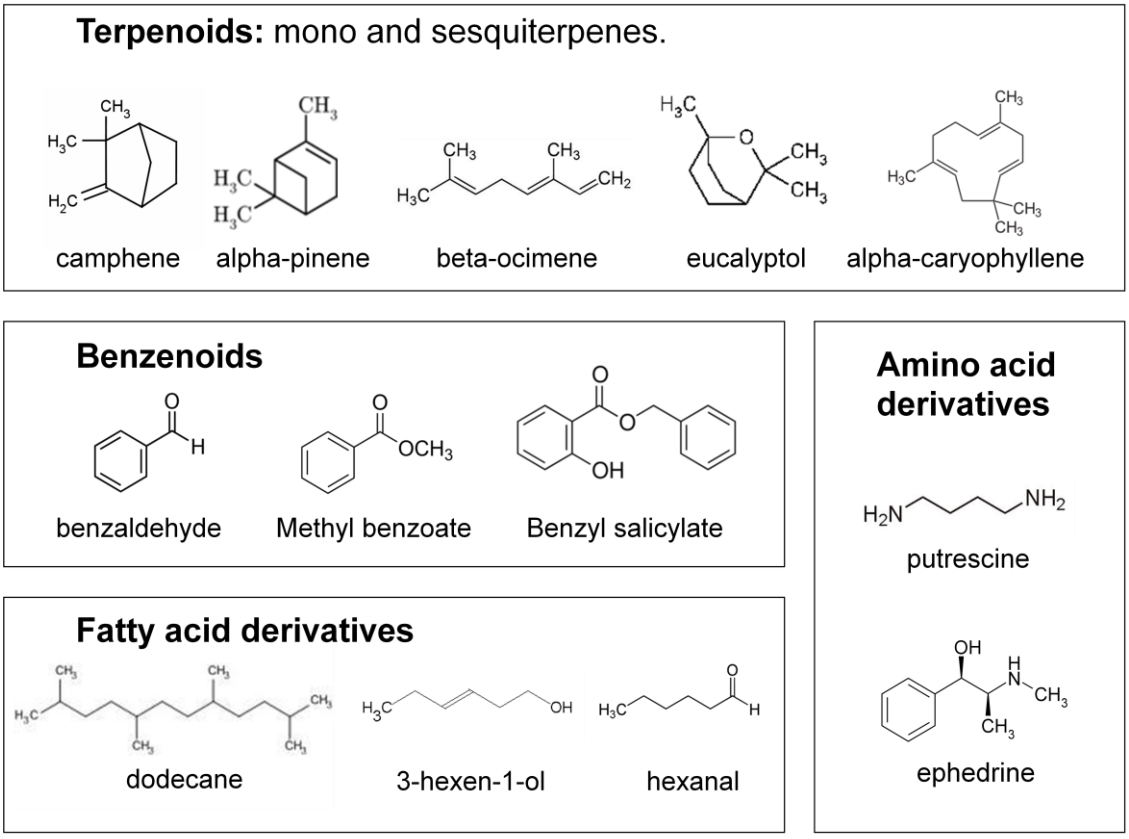

Figure 1. Plant VOCs can be mainly classified in the chemical groups of terpenoids, benzenoids, fatty acid derivatives and amino acid derivatives.

BVOC emissions develop multiple relevant roles on the abiotic and biotic environment of plants (Owen & Peñuelas, 2005; Dudareva *et al.*, 2006; Kegge & Pierik, 2010). They provide protection against several environmental conditions and stresses (Peñuelas & Llusià, 2003; Niinemets *et al.*, 2010). An example is found in the thermotolerance effect attributed to isoprene and monoterpene emissions (Sharkey & Singsaas, 1995; Chen & Cao, 2005). Volatile isoprenoids have been suggested to confer photoprotection in photosynthetic plant tissues in conjunction with non-volatile compounds such as carotenoid pigments and tocopherols (Peñuelas & Munné-Bosch, 2005).

BVOCs, and especially terpenoids, also develop defensive functions against insect herbivores and fungal pathogens (Pare & Tumlinson, 1999; Cheng *et al.*, 2007). Terpenoids are the most abundant and structurally diverse group of BVOCs, with relevant roles in direct and indirect plant defense (Cheng *et al.*, 2007; Yu & Utsumi, 2009). Several plant species, such as most conifers, accumulate terpenoids in specialized structures, such as ducts, glands and cavities for defensive purposes (Phillips & Croteau, 1999; Gershenzon *et al.*, 2000).

Floral VOCs: olfactive signals for pollinator attraction

Among those BVOC functions that are related with biotic agents we may highlight the attraction of pollinators to flowers, which is usually complemented with visual stimuli and the offer of rewards that are associated with these stimuli (Chittka & Raine, 2006; Wright & Schiestl, 2009). The distribution of pollinator visits to flowers is strongly determined in both time and space by competition and facilitation phenomena among co-occurring plants (Duffy & Stout, 2011). At this point, floral VOCs play a significant role by mediating flower location and enhancing their attractiveness to pollinators (Majetic *et al.*, 2009a).

Floral blends are mainly composed of terpenoids and benzenoids (Knudsen *et al.*, 2006a; van Schie *et al.*, 2006). Flowers attract pollinators by taking advantage of their species-specific innate preferences for certain compounds and their abilities to learn particular VOC mixtures (Farina *et al.*, 2007; Raguso, 2008; Arenas & Farina, 2012). Some floral BVOCs are considered to be generalist attractants of a wide range of pollinators (Li *et al.*, 2008; Johnson & Hobbhahn, 2010) while some others appear to act as specific attractants of particular insect species (Eltz *et al.*, 1999; Schiestl *et al.*, 2003; Schiestl & Glaser, 2012). Floral emissions provide pollinators with chemical cues (odour plumes) that serve to locate flowers and provide information about abundance and quality of floral rewards (Howell & Alarcón, 2007; Wright *et al.*, 2009) and the developmental stage of flowers (Proffit *et al.*, 2008; Goodrich & Raguso, 2009).

State of the art and recent advances in the study of floral VOCs

Plant VOC emissions have been largely studied, providing a good basis on the multiple factors that affect their emission rates. Although, to date, most of the works studying plant VOCs focused on the emissions of leaves (Peñuelas & Llusia, 2001; Blande *et al.*, 2010; Niinemets *et al.*, 2010; Llusia *et al.*, 2011). Significant advances in the research field of floral VOC emissions have been conducted in the last decade, opening new questions that need to be addressed and revealing that floral VOC emissions can also be affected by diverse biotic and abiotic factors (Effmert *et al.*, 2008; Sagae *et al.*, 2008). We count with a good conceptual framework on how floral scents are produced and emitted (Dudareva & Pichersky, 2000; Pichersky *et al.*, 2006; Muhlemann *et al.*, 2014). We also have a good knowledge on the characteristic floral emissions of several plant species, although the representativeness of these species is very unbalanced among different plant families (Knudsen *et al.*, 2006b). A remarkable limitation of most studies, however, is that they provide a detailed description of what they assume it is the characteristic floral scent blend of a particular species, by using measurements conducted at one specific moment under very specific conditions. They often rely on the fact that floral scent is mostly determined by genotype, being the result of genetic adaptation to pollinator-mediated natural selection. In fact, some traits of the particular floral scents of the species, such as the array of compounds that they are able to produce and their potential biosynthetic and emission capacities, can be evolutively determined by factors such as the mode of pollination of the species (Andersson *et al.*, 2002; Magalhães *et al.*, 2005) or their flowering phenology (Filella *et al.*, 2013). But one issue that appears to be less considered and needs to receive more attention is that phenotypic plasticity may also happen as a result of the effects of environmental conditions on plant physiology and VOC physicochemistry, and that this plasticity can play an important effect on floral emissions (Majetic *et al.*, 2009b). Most of the variables that can affect floral emissions still need to be elucidated and their effects measured.

VOC collection and analytical methods

There exist diverse options to collect VOCs depending on the kind of results that we want to obtain (Tholl *et al.*, 2006; Stashenko & Martínez, 2008). There are extractive methods that serve to conduct measurements of VOC internal contents from sampled tissues. In the case of floral scent research, they are applied to excised flowers and provide an exhaustive recovery of volatiles from floral tissues. On the other hand, headspace methods trap the volatiles from the air surrounding the sample and provide a good image of the VOC emission profile. Headspace methods can be classified in static and dynamic headspace. In static headspace, a liquid or solid sample is sealed into a vessel, where VOCs from the sample are concentrated up to reaching a steady state between the gas phase and the solid/liquid phase. An adsorbent trap can be put into the vessel to trap the volatiles or alternatively a sample volume from the air phase can be collected for posterior analysis. On the other hand, dynamic headspace sampling methods enclose the emitter sample into a chamber with a flow of carrier gas. Instead of allowing the sample to come to equilibrium in a sealed container, the surrounding air is constantly removed from the sample chamber and is trapped in an adsorbent material or analysed in real time. Dynamic headspace is more often used in floral scent research than static headspace, because it allows the quantification of emitted amounts of VOCs for comparative purposes and the calculation of floral VOC emission rates. Alternatively, static headspace is suited for qualitative analyses of VOCs and surveys of VOC profiles at a single time point, especially for low-emitting plant species, because this method enriches volatiles in the sample headspace enhancing the detection of VOCs emitted in low ratios, and also avoids sampling impurities of a continuous air stream.

VOCs are analyzed by different methods, being gas chromatography-mass spectrometry (GC-MS) the most often used in floral scent studies (Knudsen *et al.*, 2006b). Plant VOCs trapped on adsorbing matrices are routinely analyzed by the standard technique of GC-MS, which consists on the separation of volatile compounds in a GC column and their posterior detection by mass spectrometry (MS). Total ion chromatograms are obtained, which provide information on the retention time of each compounds and its mass spectrum consisting of a characteristic ion fragmentation pattern. Some experiments address questions for which continuous real-time analysis of VOCs is required. Proton Transfer Reaction-Mass Spectrometry (PTR-MS) allows fast detection of most VOCs in combination with low detection limits (10-100 pptv), thus providing the possibility to conduct continuous measurements of VOC concentrations with few seconds or minutes between each measurement (depending on the number of masses to scan). However, one limitation of PTR-MS is that it does not differentiate compounds with the same parent mass, such as monoterpenes, whose emissions are calculated together. Although PTR-MS has been used primarily for field measurements of atmospheric air composition (Hewitt *et al.*, 2003), this technique has also become a useful tool for the analysis of plant VOC emissions in addition to GC-MS analysis.

Objectives of the thesis

The main objective of this thesis is to broaden the current knowledge on the factors that determine or modify the VOC emissions of flowers, considering the possible effects of such factors on the plant-pollinator relationship.

Chapter 1 consists of a review summarizing the main functions of floral volatile emissions, analyzing the multiple sources of spatial and temporal variability in floral emissions, predicting responses of floral emissions to Global Change and pointing to future lines of research in the field of floral VOC emissions.

In the following part of the thesis we analyzed species traits that have exerted strong selective pressures on floral VOC emissions, strongly influencing and determining floral emission capacity, such as the pollination mode (chapter 2) and the flowering phenology (chapters 3 & 4).

Chapter 2 analyzed the differences in floral VOC chemical diversity and emission rates for different chemical groups between wind- and insect-pollinated flowering plant species.

Chapter 3 tested whether seasonal changes in the level of competition among coexisting flowering plants in a community for pollinator services, resulted in selective pressures for higher volatile emissions and floral rewards' production in that moment of stronger competition.

Chapter 4 described the temperature responses of floral VOC emissions, and revealed that the species-specific optimum temperature for floral VOC emissions is adapted to the temperature range during the flowering period of the species.

In the later chapters we revealed some of the effects that different biotic (chapters 5 & 6) and abiotic agents (chapters 7 & 8) can cause on floral VOC emissions.

Chapter 5 analyzed the effect of floral microbiota on floral VOC contents and emissions.

Chapter 6 analyzed the effect of florivory and folivory on floral VOC emissions.

Chapter 7 analyzed the changes in floral VOC emission rates and relative composition that occur in response to temperature changes, and predicted future changes in floral scent that may occur under projected temperature increases due to Global Warming.

Chapter 8 analyzed the effect of ambient ozone on the degradation and modification of floral volatile cues, as well as the related responses of pollinators to the altered chemical cues.

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Chapter 1. Floral volatile organic compounds: Between attraction and deterrence of visitors under global change

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Abstract

Plants produce and emit a large variety of volatile organic compounds that play key roles in interactions with abiotic and biotic environments. One of these roles is the attraction of animals (mainly insects) that act as vectors of pollen to ensure reproduction. Here we update the current knowledge of four key aspects of floral emissions: (1) the relative importance and interaction of olfactory signals and visual cues, (2) the spatial and temporal patterns of emission in flowers, (3) the attractive and defensive functions of floral volatiles and their interference, and (4) the effects of global change on floral emissions and plant–pollinator interactions. Finally, we propose future lines of research in this field that need to be addressed or investigated further.

Keywords: Flower scent, Odour signal, Pollinator attraction, Floral defence, Flower–pollinator interaction.

Introduction

Previous reviews on floral emissions have provided a good basis on the biochemical processes involved in the interactions of flowers with their flower visitors (Dudareva *et al.*, 2000; van Schie *et al.*, 2006), their action over pollinators' behaviour (Riffell, 2011) and the ecological processes that drive their evolution (Raguso, 2008a). Here we update previous reviews on floral BVOCs and address diverse complementary and less considered ecological aspects of floral volatile emissions. We review their coexistence and association with visual signals, their patterns of emission and their underlying causes, their attractive and defensive functions and their interference, and finally we discuss the potential effects of global change on plant–pollinator interactions through the induction of changes in these floral emissions.

Plants produce and emit a large array of biogenic volatile organic compounds (BVOCs) that are useful in their interactions with their immediate environment. BVOCs include terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives, and amino acid derivatives (Dudareva *et al.*, 2004, 2006). These emissions of BVOCs to the atmosphere have significant biological effects on the relationships of plants with other organisms and also environmental effects on atmospheric physicochemical properties (Peñuelas & Llusià, 2003; Peñuelas & Staudt, 2010). These volatile substances serve diverse functions in plants, including interactions with both abiotic (Sharkey & Singsaas, 1995; Peñuelas & Llusià, 2002; Peñuelas & Munné-Bosch, 2005; Niinemets, 2010) and biotic factors (Peñuelas *et al.*, 1996; Pichersky & Gershenzon, 2002; Dudareva *et al.*, 2006; Kegge & Pierik, 2010; Seco *et al.*, 2011). As sessile organisms, plants do not have the capacity to move to escape from detrimental organisms and conditions to which they are exposed. Plants have therefore evolved a great diversity of chemicals to deal with those detrimental factors. BVOCs, and especially terpenoids, are among the most relevant compounds used by different tissues of the plant to interact with their abiotic and biotic environments (Peñuelas & Llusià, 2004; Schiestl, 2010). Benzenoids are ubiquitous in floral scents (Knudsen *et al.*, 2006) and they are similarly important and abundant than terpenes (van Schie *et al.*, 2006).

This capacity to chemically interact with their environment emerged early and diversified extensively in the evolution of the plant kingdom (Chen *et al.*, 2011; Paul & Pohnert, 2011). The protection of plant tissues from its consumption by other organisms (herbivory) might be one of the first needs that the ancestors of terrestrial plants had to cope with (Van Donk *et al.*, 2010). One of the mechanisms that plants have evolved to resolve this need was the production and eventual release of deterrent compounds from their tissues. Also

competition has been one of the most common biotic interactions experienced by plant ancestors, and in response to this they have evolved allelopathic substances (Rasher *et al.*, 2011). Primitive BVOCs may have served diverse other functions related to the interaction with abiotic agents, as primitive plants have been exposed to diverse environmental stresses. With the appearance of terrestrial plants and phanerogams, diverse plant lineages developed other biological interactions, like those established with pollinators (Bronstein *et al.*, 2006). The establishment of these interactions is mediated at least in part by chemical communication channels (Negre-Zakharov *et al.*, 2009). At this point, the large array of pre-existing chemical substances may have assumed new biological functions, such as the attraction of pollinators (Pellmyr & Thien, 1986; Armbruster, 1997; Schiestl, 2010), which is one of the most relevant functions of BVOCs (Dudareva *et al.*, 2006). The evolution of these compounds experienced a new impulse with the radiation of flowering plants, as it has been stated that biotic pollination has contributed to the diversification of flowering plants and their floral traits (Grimaldi, 1999; van der Niet *et al.*, 2011).

More than 85% of the species of flowering plants depend on insects for pollination (Ollerton *et al.*, 2011). Pollinators see communities of flowering plants as "biological markets" that offer a wide variety of flowers from which they can choose those with the best rewards (Chittka & Raine, 2006). The distribution of visitors among flowers is strongly affected by competition between plants, mechanisms of facilitation for the attraction of pollinators (Ghazoul, 2006; Duffy & Stout, 2011), and competition between pollinators for the exploitation of floral resources (Pleasants, 1981). Plants need to attract and compete for the attention of pollinators to receive their services. At this point, floral recognition by pollinators plays a key role in plant–pollinator systems.

Olfactory vs. visual cues

Floral recognition is mainly mediated by colour vision and olfaction (Chittka & Raine, 2006). The visual and olfactory display of flowers includes thus the floral traits that play the most important roles in the attraction of pollinators (Kunze & Gumbert, 2001). Plant–pollinator relationships have been historically regarded to be mostly mediated by vision. The study of communication between plants and pollinators has therefore focused mostly on visual traits; little consideration has been given to the contribution of the chemical traits of floral phenotypes (Raguso, 2008b). Visual cues, though, may act in concert with olfactory cues to allow pollinators to find plants (Burger *et al.*, 2010; Leonard *et al.*, 2011a, 2011b). The presence of floral odours may enhance the discrimination of colours by improving attention towards visual cues, and the combination of chromatic and aromatic cues may enhance the formation and retrieval of memories in pollinators (Kunze & Gumbert, 2001). The relative importance of each sense may vary in the various plant–pollinator interactions. Olfactory signals are particularly important in plants that bloom at night when visual characteristics are less important for their pollinators (Jürgens *et al.*, 2002; Carvalho *et al.*, 2012); however, some nocturnal pollinators may rely in both visual and olfactory cues to locate and feed on night-blooming flowers (Raguso & Willis, 2005). In fact, investment in the production of scent as an advertisement of reward provides a net fitness benefit to plants (Majetic *et al.*, 2009a). Olfactive signals can constitute a more reliable signal for pollinators to detect the presence of rewards and find them than visual traits (Raguso, 2004a). Ample evidence shows that pollinators such as bees are able to detect pollen and nectar in flowers via olfactive cues (Wright & Schiestl, 2009, and references therein). Floral scents thus occupy a relevant place in the hierarchy of stimuli that drive floral selection (Parachnowitsch *et al.*, 2012); honey bees and bumble bees learn odours faster and with a higher retention than colours, and odours evoke a stronger discrimination between flowers (Kugler, 1943; Menzel, 1985; Dobson, 1994;

Leonard *et al.*, 2011a, 2011b). Many pollinators learn the particular scents of different species of plants to recognise those flowers offering the highest quality rewards (Chittka *et al.*, 1999). The learning of olfactory cues in pollinators strongly contributes to forming the networks of interactions established in plant–pollinator communities, which are dynamic in time and space (Riffell, 2011), and represents an important component of the selective environment determining the evolution of floral signals through their impact on plant fitness (Wright & Schiestl, 2009).

Spatial and temporal variation of floral emissions of BVOCs

The scent of different floral organs

Certain BVOCs are commonly emitted by both flowers and vegetative parts of plants (Dudareva & Pichersky, 2000). Some compounds produced only by the flower, however, may serve flower-related functions, such as the attraction of pollinators or the deterrence of nectar thieves. Different floral parts such as petals, sepals, pollen, and nectar can emit diverse blends of BVOCs (Dötterl & Jürgens, 2005; Mena Granero *et al.*, 2005; Jullien *et al.*, 2008; Filella *et al.*, 2011). These blends may serve different functions developed by diverse floral organs. Some organs may preferentially attract the attention of visiting animals or present particular chemical defences. In many cases, differential emission patterns along different flower organs serve pollinators to find reward-offering structures (Flamini *et al.*, 2002; Dötterl & Jürgens, 2005).

Petals and sepals

In many species of plants, scents from whole flowers are predominantly composed of volatiles emitted from petals, mainly benzenoids, phenylpropanoids, nitrogen-bearing compounds, and terpenoids, such as the common floral monoterpene β-ocimene (Bergström *et al.*, 1995; Dötterl & Jürgens, 2005; Mena Granero *et al.*, 2005; Knudsen *et al.*, 2006). Petals, though, are not always the only organs of flowers with the highest emissions (Mactavish & Menary, 1997a; Jullien *et al.*, 2008).

While leaves store BVOCs in a variety of different structures (trichomes, idioblasts, cavities, and ducts), depending on the species (Werker, 1993; Gershenzon *et al.*, 2000; Turner *et al.*, 2000; Gang *et al.*, 2001), flowers usually produce their blends of BVOCs in osmophores or in conical cells located in the petals (Bergougnoux *et al.*, 2007; Whitney *et al.*, 2011) and sometimes in other floral structures such as sepals (Cabral *et al.*, 2010). Vogel (1962) established the term osmophore for an enclosed area of floral tissue that specialises in the emission of scents. Osmophores consist of a multilayered glandular epithelium (Vogel, 1962; Stern *et al.*, 1987; Hadacek & Weber, 2002). Cabral *et al.* (2010) found evidence that the volatiles in species of *Acianthera* are released by the cells of the osmophores and stored in periplasmic and intercellular spaces. They suggest that these compounds are probably volatilised by daytime temperatures and are released through the stomatal pores in sepals. Petal emissions have been observed to correlate well with endogenous concentrations along floral maturation, which leads to the conclusion that at least in many cases petal emissions are released more or less readily depending on their volatility and their internal concentration (Bergougnoux *et al.*, 2007). The diffuse emission of BVOCs is probably a plesiomorphic character of flowers, while the spatial pattern of emission, characterised by the distribution of osmophores, is most likely an apomorphic character (Vogel, 1962; Bergström *et al.*, 1995). In

some cases, concentration gradients of BVOCs along petals indicate the path to reach floral nectaries (Bergström *et al.*, 1995; Dötterl & Jürgens, 2005).

Pollen

The characterisation of chemical constituents present in the odour of pollen has been largely ignored because of difficulties in sampling and analysis. Samples of odours from pollen analysed with headspace techniques have been found to be chemically different from scent from whole flowers (Dobson *et al.*, 1996; Flamini *et al.*, 2002), and the diversity of compounds identified is often lower in pollen (Knudsen & Tollsten, 1991; Dobson *et al.*, 1996). Odours from pollen are probably detected at short distances by insects in those cases in which pollen emissions of BVOCs are quantitatively less abundant than those from the entire flower. However, many species may present stronger pollen odours than others (Dobson & Bergström, 2000).

Plants with pollen that has an odour significantly different from that of other floral structures are able to advertise the existence of pollen as a reward, providing these plants with an additional level of specific differentiation from other plants. The benefits offered by plants that present a characteristic odour from pollen may include providing pollen-foraging insects with a higher efficiency to locate food, and a higher fitness for the plant due to an increased export of pollen and the more effective transport of pollen to stigmas (Dobson & Bergström, 2000). Pollen applies two main pressures on plants: the need for protection from non-pollinating insects that exploit it without providing any benefit for the plant (Hargreaves *et al.*, 2009), and the need to make this reward more attractive to pollinators. This conflict may play a key determining role in the evolution of pollen's odour and of other floral traits, such as nectar, that also suffer from over-exploitation by non-pollinating insects.

Knoll (1930) was the first to study the origin of odours from pollen and proposed that scents came from the pollenkitt. This term refers to the oily, and often sticky, coloured substances coating pollen grains that cluster the grains into aggregates. Knoll (1930) enumerated and extensively discussed the diverse functions attributed to the pollenkitt, among which are those that enhance adhesion, confer the yellow pigmentation that protects pollen from UV radiation, provide nutritional value through fatty oils, and confer odour. Pollenkitt thus has a large variety of functions in addition to the provision of odour (Dobson, 1989; Pacini & Hesse, 2005), which may imply the application of a great diversity of evolutionary pressures driving the traits of the pollenkitt. Dobson *et al.* (1987) confirmed that all the main compounds found in pollen headspace can be found in the pollenkitt. Measurements conducted by Dobson *et al.* (1990) suggested that the pollenkitt adsorbs some volatile compounds from the surrounding air, which is impregnated with diverse odours from petals.

Pollen constitutes an important nutritional resource for many insects that visit flowers, especially as a source of proteins and lipids (Roulston & Cane, 2000). Some studies have investigated how signals from pollen (basically olfactory and visual) affect the localisation and identification of pollen by insects (Lunau, 1992; Dobson & Bergström, 2000). The need to explore the behavioural responses of insects to BVOCs constitutes an added difficulty of this field of research. Various studies provide evidence that pollen-seeking insects such as bees (Dobson *et al.*, 1999; Dobson & Bergström, 2000), beetles (Cook *et al.*, 2002; Bartlet *et al.*, 2011), and syrphids (Golding *et al.*, 1999) rely on gradients of odour during their search for food. BVOCs from pollen may be required to induce pollinators to land on flowers. An example is found in foraging-naive bumble bees, in which landing is most effectively elicited when

combining olfactory signals from pollen with visual stimuli from anthers (Lunau, 1992). Dobson *et al.* (1999), in a series of behavioural field studies of bumble bees foraging for pollen on *Rosa rugosa*, provided the strongest evidence that bees use scents from pollen to distinguish between flowers that have different amounts of pollen.

Nectar

In many plants, pollination requires the help of nectar-feeding pollinators. Floral nectar is the most common reward that plants offer to their pollinators (Simpson & Neff, 1983). The chemical composition of nectar is complex; it contains primary metabolites, such as sugars and amino acids that are used to attract pollinators, but also secondary metabolites, such as alkaloids, phenolics, and nonprotein amino acids (Baker, 1977) that repel nectar thieves and also have undesired negative effects on pollinators' visits (Stephenson, 1981; Kessler & Baldwin, 2007). Although we know that the sugars in nectar come from nectaries (Fahn, 1979), little is known about the origin of the secondary metabolites found in nectar. The set of BVOCs present in nectar comes both from the volatiles released by the surrounding floral tissues and from those released by the nectaries into the nectar solution (Balao *et al.*, 2011). Raguso (2004b) found that some volatile compounds were taken up by artificial nectar applied to petals of *Magnolia grandiflora*, while others were not. Volatile compounds providing information about the existence of nectar often come from other flower structures, such as the above-mentioned nectar guides present in petals and other floral organs. The function of those BVOCs associated with the presence of nectar is probably complex, given that these scents have both attractive (Honda *et al.*, 1998; Raguso & Willis, 2002) and deterrent effects on nectar consumers (Raguso & Willis, 2002).

Spatial diffusion and distribution of floral scent

Once BVOCs are released from flowers, they are rapidly mixed and diluted by physical atmospheric processes creating a dynamic olfactory environment (Riffell *et al.*, 2008). They generate different patterns of diffusion depending on which type of transport predominates, advection or turbulence. Advective transport generates a continuous concentration gradient near the source of emission, while turbulent transport creates filaments of odour of intense concentration. Pollinators may perceive these scent trails differently and may adapt their navigational strategies (Cardé & Willis, 2008) and sensory systems to them. Moreover, the dynamics of odours may be affected by the size and position of the source of emission above the substrate. The size of the sources (flowers) and their position relative to the ground, then, may be floral traits available to selection via patterns of diffusion. These traits elicit different efficiencies in the detection and location of a signal's source by the pollinator. Atmospheric dynamics influencing the distribution of odours vary among different habitats (e.g. grassland, shrubland, or dense forest). Habitats to which plants and insects are adapted may exert diverse selective pressures on floral traits and the sensory systems of pollinators through the influence of atmospheric dynamics and the diffusion of odours. The environment of odours experienced by animals is also affected by their own body size and translational speed (Riffell *et al.*, 2008).

The distance at which BVOCs can be perceived may depend on both the physicochemical traits of the BVOCs and the sensitivity of the sensory systems of the pollinators (Chittka & Raine, 2006; Riffell, 2011). BVOCs have different chemical properties that confer different reactivities and longevities (Atkinson & Arey, 2003). These properties

affect the persistence of BVOCs in the air and their capacity to be transported large distances from their source (Blande *et al.*, 2010)(Blande *et al.*, 2010).

Temporal variation in floral scent

During their lifespan, flowers may vary their scent both quantitatively and qualitatively for many reasons. Temporal patterns of emission can become apomorphic traits that reflect convergent evolution based on particular relationships with certain groups of pollinators (Morinaga *et al.*, 2008; Okamoto *et al.*, 2008; Dötterl *et al.*, 2012). Plants may benefit from emitting higher amounts of BVOCs when the principal pollinator is active and also from saving resources by reducing emissions when the pollinator is not active. These factors may affect the circadian variation of such floral emissions, as occurs in moth-pollinated flowers, emitting maximal scent in early evening and night (Raguso *et al.*, 2003; Okamoto *et al.*, 2008), although not always (Pichersky *et al.*, 1994). Apart from the conservation of resources, a reduction in volatile emissions when specialist pollinators are inactive may partially prevent the visitation of generalist pollinators, which can result in less effective pollination. Nevertheless, when specialist pollinators are rare, plants may take advantage of visits from generalist pollinators. Plants that mainly attract specific pollinators may change floral blends to attract generalist pollinators and ensure pollination when flowers remain unpollinated for a long time (Dudareva & Pichersky, 2000). Individual flowers may also change or reduce their emissions once they are pollinated (Negre *et al.*, 2003) to prevent more visits that can cause damage to flowers, direct visits to flowers that are still unpollinated (Rodriguez-Saona *et al.*, 2011), and prevent visits from florivores or seed-feeders (Muhlemann *et al.*, 2006). Floral emissions may vary with floral ontogeny due to differences in floral processes and stages of maturity of different floral parts (Mactavish & Menary, 1997b). In plants with unisexual flowers, emissions may vary between male and female flowers with floral maturation (Proffit *et al.*, 2008; Ashman, 2009). Many hermaphroditic flowers may experience changes in their scent profile during their lifetime due to a temporal differentiation in the male and female phases (Goodrich *et al.*, 2006; Goodrich & Raguso, 2009). The emission of BVOCs by plants, including floral scents, may represent a heritable component of phenotypic plasticity that may be species (or population) specific and may be modulated by environmental conditions (Majetic *et al.*, 2009b).

Functions of floral scent: attraction

Floral scents are composed of a mixture of BVOCs with characteristically high vapour pressures and low molecular weights (Copolovici & Niinemets, 2005; Knudsen *et al.*, 2006). Benzenoids, whose biosynthetic pathways are related to the synthesis of floral pigments mainly found in floral tissues (van Schie *et al.*, 2006), can serve attractive functions in flowers, while various terpenoids, which can be also found in emissions from vegetative tissues, have both attractive and deterrent effects on facultative visitors. Pollinators and other visitors may exert different selective pressures on flowers that may affect quantitative and qualitative differences in floral blends of BVOCs. To date, many studies have addressed the difficult task of revealing the differential traits of floral scents that are typical of particular pollination syndromes (Knudsen & Tollsten, 1993; Andersson *et al.*, 2002; Raguso *et al.*, 2003; Pettersson *et al.*, 2004; Dobson, 2006).

Most pollinators rely on floral rewards offered by flowers, such as nectar, pollen, or oil products (Steiner *et al.*, 2011). Floral BVOCs provide information about the location, abundance, and quality of floral rewards (Howell & Alarcón, 2007; Wright *et al.*, 2009). Flowers attract pollinators by exploiting their species-specific innate preferences and their abilities to

learn the association between scent and floral reward (Farina *et al.*, 2007; Raguso, 2008a; Arenas & Farina, 2012). Plants that do not offer nectar may mimic the scents and colours of neighbouring flowers that do attract pollinators (Kunze & Gumbert, 2001; Schiestl, 2005). While some BVOCs are known to be generalist attractants of a great diversity of pollinators (e.g. Li *et al.*, 2008; Johnson & Hobbhahn, 2010), others may be specific attractants of particular species of insect (e.g. Eltz *et al.*, 1999; Schiestl *et al.*, 2003; Schiestl & Glaser, 2012). For example, some orchids attract only males of their pollinator species by emitting analogues of feminine pheromones, thereby tricking the males into believing that the flowers are females (sexual deception) (Schiestl, 2005; Gaskett, 2011; Gögler *et al.*, 2011). Some flowers deceive insects that feed on other animals by emitting BVOCs that mimic prey-related odours (Shuttleworth & Johnson, 2009; Stökl *et al.*, 2010) or the emissions of herbivore-infested plants (Brodmann *et al.*, 2008, 2012). Other flowers emit volatile blends that resemble the odours of carrion or dung to attract pollinators (Urru *et al.*, 2011; van der Niet *et al.*, 2011). Male euglossine bees are attracted to and collect floral volatiles from particular species of orchids to attract females (Eltz *et al.*, 1999; Embé, 2004). Some of these relationships are very specific and rare but together highlight the capacity of floral scents to attract pollinators by providing information about very different types of resources, even though these may not exist. Most of these plant–pollinator interactions involving only one plant species and one or few species of insects are mediated by private communication channels, consisting on the emission of unusual BVOCs, the emission of specific ratios of more ubiquitous compounds, and the use of volatiles that act as filters of particular floral visitors (Raguso, 2008a).

The importance of BVOCs in the ecology of several insect groups suggests that selective pressure on floral scent by pollinators is widespread in entomophilous plants (Schiestl, 2010). Entomophilous plants (pollinated by insects) emit more pronounced scents than ornithophilous (pollinated by birds) and anemophilous plants (pollinated by wind) (Magalhães *et al.*, 2005; Wragg & Johnson, 2011). This tendency suggests that emission of BVOCs from flowers evolved mainly to attract insect pollinators. Dobson (1988) observed that species pollinated by insects (e.g. Lepidoptera) that consume rewards other than pollen tended to have pollen with relatively fewer BVOCs than species pollinated by bees that rely more on pollen as a food source.

Functions of floral scent: defence

Plants may experience detrimental effects from being visited by some non-pollinating flower visitors that consume floral rewards (Urkle *et al.*, 2007), disturb pollinators (Tsuji *et al.*, 2004; Junker *et al.*, 2007), or feed on floral tissues (McCall & Irwin, 2006). The detrimental effects caused by antagonistic visitors can exceed the benefits from mutualists (Morris *et al.*, 2007). Plants may benefit from selecting visitors of flowers, and they present a variety of defensive properties that include some compounds of floral scents (Kessler & Baldwin, 2007; Junker & Blüthgen, 2008; Kessler *et al.*, 2008; Willmer *et al.*, 2009; Galen *et al.*, 2011). Junker & Blüthgen (2010) demonstrated that some floral BVOCs act as “filters” that select effective pollinators and deter detrimental (thieves and herbivores) and neutral floral visitors (generalist pollinators carrying heterospecific pollen). From this viewpoint, obligate visitors, either mutualistic or antagonistic, may have evolved a tolerance to deterrent and toxic compounds present in floral structures and scents and can use these compounds as specific signals to find their host plants. Some floral volatiles have antimicrobial properties to protect floral structures by preventing colonisation by bacterial communities that can alter floral tissues and the chemistry of nectar (Tholl *et al.*, 2005; Junker *et al.*, 2011).

Defensive functions of the odour of pollen

The presence of volatile compounds in pollen, the variation in their composition and relative abundance, and the repulsive and antimicrobial function of some of these compounds suggest that BVOCs of pollen have additional functions besides attracting pollinators. One of the main functions of these compounds is to protect the male gametophyte from pollen-consuming animals and pathogens that do not provide any benefit to plant fitness. BVOCs that repel thieves may be found in diverse floral parts (Mullin *et al.*, 1991). Plants with deterrent chemicals in non-pollen floral parts might avoid the need to have them in the pollen. Anthers may repel unwanted visitors that consume pollen by presenting deterrent compounds (Belcher *et al.*, 1983; Rossiter *et al.*, 1986). Conversely, attractive volatiles released by sterile pollen of heterantherous flowers (Faden, 2008) or by highly attractive food structures, such as staminodes (Bergström *et al.*, 1991; Endress, 1994), may keep pollen-feeders away from the fertile pollen. The two abundant α-methyl ketones in the odour of pollen in *R. rugosa*, namely 2-undecanone and 2-tridecanone (Dobson *et al.*, 1990), are deterrent and even toxic to several insects (Kennedy *et al.*, 1991; Maluf & Barbosa, 1997), and some α-methyl ketones have antifungal activity (Cole & Blum, 1975). Anemophilous plants are not expected to suffer disadvantages from presenting deterrent compounds in their flowers, because they do not need to attract pollinators (Dobson & Bergström, 2000). They are then expected to present more chemical defences in the scent of their pollen.

Some BVOCs in pollen have concurrent multiple functions. A variety of essential-oil volatiles identified in the scent and considered to be attractants to pollinators might also have microbial and fungal defensive functions (Knobloch *et al.*, 1989; Kubo *et al.*, 1995). An example is eugenol, a common volatile of pollen found in *R. rugosa*, that has both the potential to attract an array of insects and antimicrobial activity (Zaika, 1988). The lactone parthenin, a sesquiterpene, has at least three functions: defence, attraction of specialist herbivores, and pollen allelopathy (Jayanth *et al.*, 1993). Pollen allelopathy is a phenomenon that has been rarely documented among the functions of BVOCs. The allelopathic effects of BVOCs in the pollen of one species deter the germination of pollen from other species, conferring a competitive advantage to the species with these compounds (Murphy, 1999). Pollen allelopathy has been documented in few species, and the magnitude of its effects on ecosystems requires investigation (Murphy, 2000).

Interference of pollination by defensive volatiles

The need to inform pollinators about the presence of floral rewards is in conflict with the potential detrimental effects of non-pollinating visitors that may also be attracted by these compounds. To solve this problem, some plants pollinated by specialists emit defensive BVOCs from flowers that act as “filters” by selecting some visitors while inhibiting others (Figure 1A; Ômura *et al.*, 2000; Junker & Blüthgen, 2010; Junker *et al.*, 2010). The emission of 2-phenylethanol by the flowers of *Polemonium viscosum* causes different responses in visitors depending on the dosage (Galen *et al.*, 2011). When released at high rates, 2-phenylethanol repels both ant thieves and pollinators, triggering negative effects on pollination and plant fitness. When released at intermediate rates, 2-phenylethanol deters thieves and reduces the consumption of nectar per visit by pollinators, enhancing the number of visits of pollinators per volume of nectar and stimulating the movement of pollinators between different flowers, which may encourage outcrossing. The presence of nicotine in nectar has also been observed to deter thieves and to optimise the number of pollinators’ visits per volume of nectar consumed (Kessler & Baldwin, 2007; Parachnowitsch *et al.*, 2012). Another frequent strategy is to present qualitatively different floral bouquets of BVOCs. While some BVOCs are efficient at

attracting pollinators (Plepys *et al.*, 2002; Cunningham *et al.*, 2004), other compounds with deterrent functions can negatively affect plant fitness by repelling pollinators (Kessler *et al.*, 2011). By assuming a heritable component of floral scent, we can expect that the evolution of floral blends of BVOCs may be driven by positive selection on BVOCs that attract pollinators and negative selection on those that repel pollinators (Schiestl *et al.*, 2011). Pollinators can exert stronger selection pressures on floral traits than herbivores or florivores (e.g. Bartkowska & Johnston, 2012). Adler *et al.* (2012) have shown that entomophilous *Nicotiana* species present lower amounts of chemical defences in their flowers than their autogamous relatives, and that floral contents of these compounds shown a good correlation with those of leaves, suggesting a pleiotropic effect among the contents in these different tissues and indicating a selective effect of pollinators on deterrent floral compounds and indirectly on leaf compounds. BVOCs that deter herbivores and thieves may also be positively selected (Figure 2).

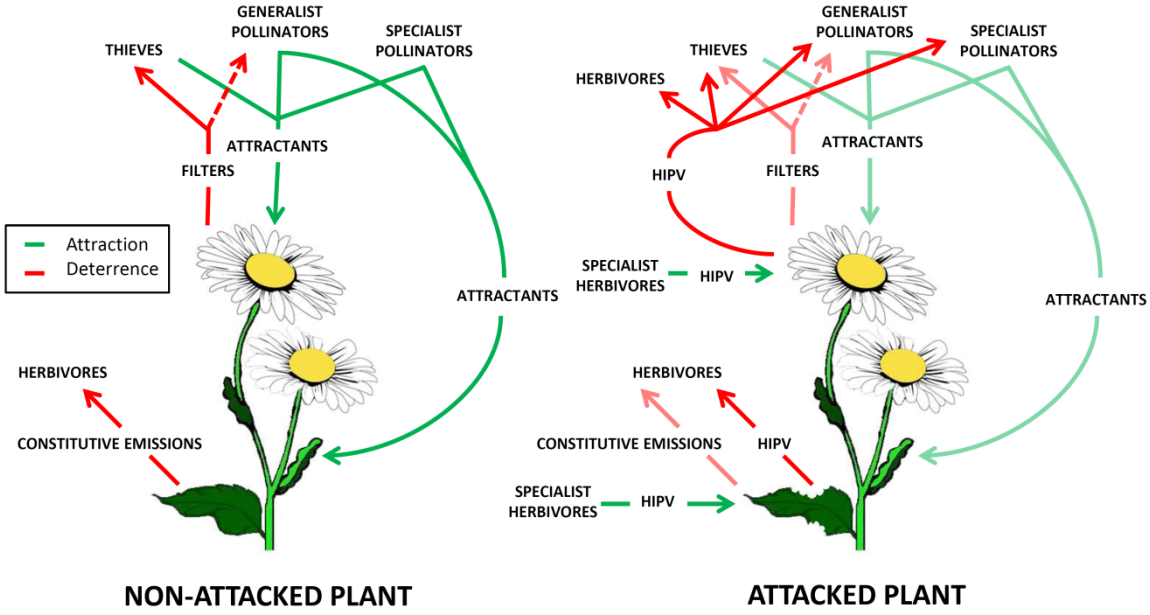

Figure 1. Effects of BVOCs on plant–pollinator interactions and the interferences of herbivory. BVOCs are classified as attractive (those related to the attraction of pollinators and therefore to plant reproduction) or deterrent (involved in plant defence and avoidance of detrimental visitors). Notice that a particular compound can cause different behavioural responses in diverse visitors belonging to different insect groups, as they have different olfactory preferences and can therefore develop both attractive and defensive functions simultaneously. This receiver-specific effect of BVOCs becomes useful for plants to select a particular visitor profile that constitutes an efficient pollination vector while keeping detrimental visitors away. Figure shows the different effects of BVOCs emitted by attacked and non-attacked plants on visitors. (A) Flowers from non-attacked plants constitutively emit BVOCs that attract a wide variety of insects, including both pollinators and thieves. Flowers from non-attacked plants may also constitutively emit specific compounds that act as filters that deter thieves and some generalist pollinators while allowing specialist pollinators to visit flowers. Leaves from non-attacked plants can constitutively emit BOVCs to deter herbivores. Constitutive emissions from leaves can also be involved in pollinator attraction (Dufaÿ *et al.*, 2003; Caissard *et al.*, 2004). (B) In flowers of herbivore-attacked plants, moreover, production and emission of herbivore-induced plant volatiles (HIPVs) may be elicited by the transduction of signals from damaged leaves to flowers (systemic response). These HIPVs may repel visitors (both pollinators and thieves) and thus interfere in pollination. Leaves and flowers from herbivore-attacked plants emit HIPVs to repel herbivores. However, specialist herbivores (monophagous and oligophagous herbivores) that have coevolved with their nutritious plants can use HIPVs to find the plants.

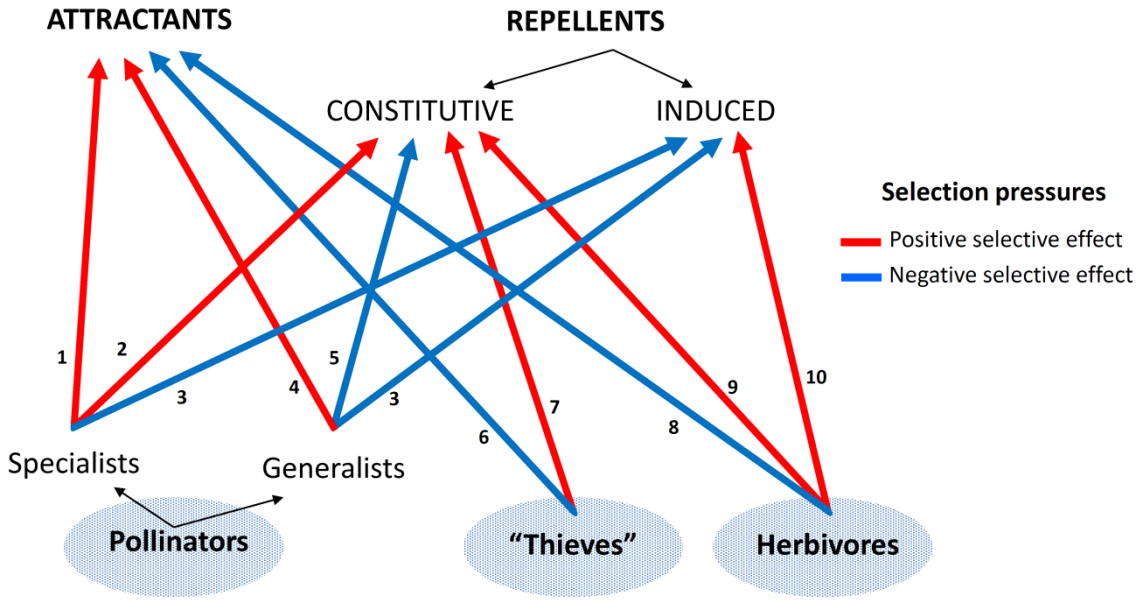

Figure 2. Selective pressures exerted by plant visitors on functional groups of BVOCs. Constitutively emitted attractive floral BVOCs are mainly selected to attract pollinators (1, 4). Attractive BVOCs, however, may have negative effects on plant fitness by attracting detrimental visitors that may consume rewards or feed on flowers and fruits (6, 8). Constitutive deterrent BVOCs from flowers are selected to repel unwanted visitors such as thieves (7) and herbivores (9). Obligate pollinators may have coevolved with the chemical defences of plants and can tolerate deterrent BVOCs and even use them to locate their host plant (2), while such BVOCs may inhibit visits by generalist pollinators (5). HIPVs are selected to deter herbivores (10). HIPVs may have detrimental effects on pollinators visiting flowers and even inhibit their visits (3). Numbers indicate the references supporting the selective pressures presented in figure: 1. Majetic *et al.* (2009a), Wright & Schiestl (2009), Filella *et al.* (2011), Wragg & Johnson (2011), Plepys *et al.* (2002), Schiestl *et al.* (2011). 2. Junker & Blüthgen (2010), Junker *et al.* (2010). 3. Adler & Irwin (2005), Kessler *et al.* (2011), Kessler & Halitschke (2009). 4. Wright & Schiestl (2009), Wragg & Johnson (2011), Schiestl *et al.* (2011). 5. Adler & Irwin (2005), Schiestl *et al.* (2011). 6. Okamoto *et al.* (2008). 7. Ômura *et al.* (2000), Junker & Blüthgen (2008), Shuttleworth & Johnson (2009), Willmer *et al.* (2009), Junker & Blüthgen (2010), Junker *et al.* (2010). 8. Theis (2006), Muhlemann *et al.* (2006). 9. Adler *et al.* (2001). 10. Adler *et al.* (2001).

The rarity of some defensive floral volatiles can provide floral scents with a higher level of specificity that favours the identification of host-plant flowers by pollinators. Examples are the array of defensive chemicals, such as the lactone protoanemonin in the odour of pollen from *Ranunculus acris* (Bergström *et al.*, 1995) and α-methyl ketones in the pollen from *R. rugosa* (Dobson *et al.*, 1990, 1999). Deterrent BVOCs might also enhance the selection of pollen by pollinators (Schmidt, 1982). Blends of BVOCs that include unusual volatiles with defensive functions may assist pollinators to become specialised on particular species, as these taxon-specific compounds may become key signals in host recognition (Junker & Blüthgen, 2010).

When leaves or flowers suffer attacks from herbivores or pathogens, the chemistry of flowers may change. Many plants may react to herbivory by inducing the production of toxins in nectar and floral tissues (Adler *et al.*, 2006; Mccall, 2006) and by producing herbivore-induced plant volatiles (HIPV) in leaves and flowers (Röse & Tumlinson, 2004; Peñuelas *et al.*, 2005; Kessler & Halitschke, 2009; Zangerl & Berenbaum, 2009). Ample evidence confirms that HIPVs can affect several members of the insect community, including pollinators, herbivores, and predators (Figure 1B; reviewed by Lucas-Barbosa *et al.*, 2011). Few studies, though, have

investigated the induction of systemic emissions of floral HIPVs after the consumption of leaves by insects and the potential effects of HIPVs on plant fitness and the behaviour of pollinators (Effmert *et al.*, 2008; Kessler and Halitschke, 2009; Theis *et al.*, 2009; Kessler *et al.*, 2011). Systemic phytochemical responses to herbivory have been observed in undamaged leaves (Turlings and Tumlinson, 1992; Mattiaci *et al.*, 2001; Rodriguez-Saona *et al.*, 2009; reviewed by Paré and Tumlinson, 1999). Damaged leaves might also induce emissions of HIPVs from flowers. Even if floral emissions did not vary in response to attacks from insects (Effmert *et al.*, 2008), HIPVs produced and emitted by attacked leaves may modify the chemical mixture of compounds in the air surrounding the plant and have the potential to alter the behaviour of pollinators visiting flowers. Systemic responses have been observed to operate in the opposite direction, from damaged flowers to undamaged leaves (Röse and Tumlinson, 2004).

BVOCs of plants can influence the foraging behaviour of pollinators (Kessler & Halitschke, 2007, 2009; Kessler *et al.*, 2008; Raguso, 2008b), and the role played by HIPVs in the behavioural changes observed in pollinators warrants further investigation (Kessler & Halitschke, 2007; Dicke & Baldwin, 2010). In *Cucurbita pepo* subsp. *texana* plants subjected to herbivory, no effects were seen on the behaviour of pollinators when herbivory caused changes in the number of flowers, display, or quality of the rewards, but visits from pollinators were reduced as a result of changes in the rates of floral emissions of BVOCs (Theis *et al.*, 2009). Phytophagous attacks can cause divergent consequences in the attraction of pollinators. Root herbivory in *Sinapis arvensis* increased the visits to flowers by pollinators (Poveda *et al.*, 2003), but the combined herbivory of leaves and roots induced a reduction in the flowering period and the number of fruits produced, although seed production was not affected. In most studies, however, herbivory of both flowers and leaves decreased visitation by pollinators (Strauss *et al.*, 1996; Adler *et al.*, 2001; Kessler & Halitschke, 2009; Cardel & Koptur, 2010; Danderson & Molano-flores, 2010). Flowers of wild tomato plants received fewer and shorter pollinator visits when the leaves of the plant were damaged by an insect herbivore (Kessler & Halitschke, 2009). These results indicate that local and systemic emissions of HIPVs may influence the foraging behaviour of pollinators, and when visitation is negatively affected, a negative selective pressure is exerted on these emissions (Dicke & Baldwin, 2010). Hare (2010) found that the production of HIPVs in *Datura wrightii* was especially high in spring during the vegetative growing phase, but production declined after the plants began to flower and produce fruit. This timing may avoid the interference between the release of HIPVs and the attraction of pollinators and seed dispersers. The induction of HIPVs during the flowering phase can have a major effect on community dynamics (Kessler & Halitschke, 2007; Poelman *et al.*, 2008). Moreover, the emission of floral HIPVs can influence different groups of the local insect community, such as nectar thieves and florivores (Baldwin, 2010).

Effects of global change on emissions of BVOCs

Environmental conditions are changing globally as a result of human activities. Changes in temperature, precipitation, land use, concentrations of atmospheric CO_2 and ozone, and UV radiation, among others, are expected to affect emissions of BVOCs by plants (Figure 3; Loreto & Schnitzler, 2010; Peñuelas & Staudt, 2010). These changes are quite variable in intensity, timing, BVOCs, and species but are generally likely to increase emissions of BVOCs (reviewed by Peñuelas & Staudt, 2010). Higher emissions might increase the efficiency of reproduction by enhancing plant–pollinator interactions. The global changes may not only induce quantitative variations in emissions, but may also cause qualitative variations in species-specific blends due to differential responses of the different compounds to the changes. Changes in the rates of emission induced by an increase in temperature can be compound specific (Llusia & Penuelas, 2000; Loreto & Schnitzler, 2010). Some BVOCs may experience a more pronounced increase in

their rates of emission than others. The rates of emission from plant tissues depend in large part on the physicochemical properties of the BVOCs, mainly their volatility. Henry's law constants (Hpc) of diverse substances respond differently to increases in temperature (Copolovici & Niinemets, 2005; Allou *et al.*, 2011). This compound-specific increase in volatility will result in an increase in the rate of emission of stored BVOCs that are also compound specific (Llusia & Penuelas, 2000). This response to temperature involves factors other than those directly related to physicochemical properties, such as factors involved in physiological responses.

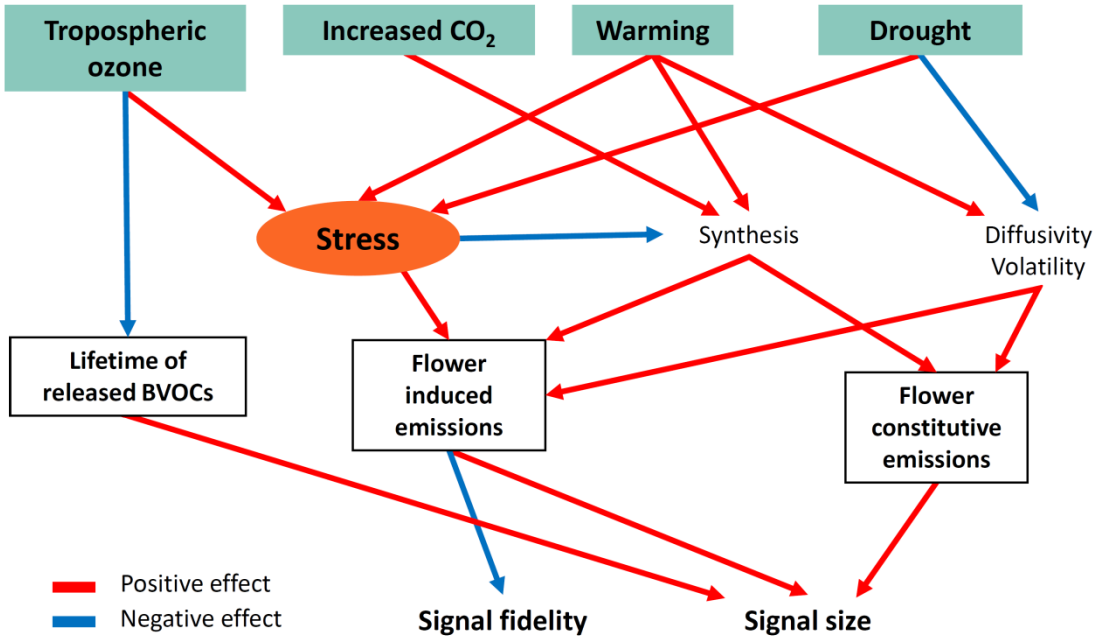

Figure 3. Effects of drivers of global change on floral odour signals. Red arrows indicate effects that become totally or mainly positive, while blue arrows indicate negative effects. Most drivers of global change (warming, drought, elevated concentrations of atmospheric CO_2 or tropospheric O_3) may cause an increase in floral emissions via stress and an elicitation of induced floral volatiles. Also, increased synthesis, diffusivity and volatility of constitutive BVOCs stimulated by higher concentrations of CO_2 and higher temperatures are expected and will be reflected in higher rates of emission of volatiles. On a longer time scale, stress may limit the capacity of plants to invest resources in the synthesis of BVOCs and can therefore reduce floral emissions. Drought may induce stomatal closure and reduce evapotranspiration in leaves and petals, which may reduce floral emissions. An increase in tropospheric ozone may reduce the longevity of BVOCs once they are released into the air and may therefore reduce the extent to which signals are detectable by pollinators. The rates of emission of different BVOCs may be affected in different ways and to different degrees by environmental conditions, and the relative proportions of volatiles present in floral blends may thus experience variations, driving changes in floral olfactory signals that can confuse foraging pollinators and result in less effective pollination, with consequent effects on reproductive success.

Rates of emission also depend on the activities of particular temperature-dependent enzymes (Monson *et al.*, 1992). The enzymatic activities of various terpene synthases may present enzyme-specific temperature curves. Even though the positive effects of temperature on the volatility and biosynthesis of BVOCs are clear, changes in temperature may be accompanied by changes in the availability of water or in other environmental conditions whose effects on rates of emission may interact with or even neutralise those of temperature (Fortunati *et al.*, 2008). In the end, though, the relative composition of a scent is likely to change as a result of all these interacting environmental changes, with likely significant

consequences for the plant–pollinator relationship. Of the interactions that may be affected by altered emissions, the plant–pollinator interaction is probably the most susceptible to interference, especially in plants that rely on only one or a few species of pollinators. This situation may have serious consequences on reproduction and the functioning of ecosystems.

In addition to warming, other components of global change varying widely on a local scale, such as air pollution and high concentrations of ozone, might induce a reduction in the efficiency of pollinators to forage for flowers by reducing the longevity of BVOCs once released and the distance at which they can be perceived by pollinators (McFrederick *et al.*, 2008; Blande *et al.*, 2010). Nevertheless, the effects of the various drivers of global change on floral emissions are difficult to predict, because they can variously affect emissions by altering different processes occurring in the plant or by interacting with BVOCs once they are released. The particular responses of plant–pollinator interactions to these altered emissions may depend on traits particular to the species of both plant and pollinator. Assuming that some plant–pollinator interactions may be more affected than others within the networks present in biological communities, changes in the emission of BVOCs will therefore probably translate into variations in the competitive abilities of pollinators and plants.

Perspectives

The unknowns highlighted in this review warrant much more research effort for the characterisation (spatial and temporal) of emission of BVOCs from different floral parts, such as sepals, carpels, and osmophores, the specialised cellular and glandular structures that produce and emit BVOCs in flowers. Some studies have confirmed that the functions of different floral organs are actually reflected in different profiles of emission. However, future studies should address the composition and function of floral scent bouquets on a finer spatial scale within the flower. Static headspace sampling techniques may serve to characterise the emission profiles of the diverse organs, including scarce compounds emitted in low rates. On the other hand, dynamic headspace sampling techniques may be indispensable to quantify total and compound specific emission rates, to compare signal size of diverse floral organs and see the particular contribution of each one to the whole floral scent. Diverse volatile sampling methods have been employed to analyse flower scent with different advantages and disadvantages over other methods (Stashenko & Martínez, 2008) that must be considered when designing an experiment.

The temporal variations of floral scent generated by floral rhythms or maturation clearly warrant further investigation. An interesting phenomenon, which needs more evidence, is the suggested capacity of the flowers of specialist-pollinated plants to shift their emissions to attract a more generalist range of visitors when they remain unpollinated for a long time (Dudareva & Pichersky, 2000).

A key question that arises from the high phenotypic variability observed in emissions of floral BVOCs (Wright & Thomson, 2005; Majetic *et al.*, 2009b) is whether this plasticity is similar in plants with different levels of selective pressure acting on floral scent (such as specialist- and generalist-pollinated plants). Less variability in the profiles of floral scents is expected in plants experiencing higher selective pressures on floral scent as a reliable signal for pollinators. Therefore, it is warranted an extensive and intensive comparison of the phenotypic variability in the ratios of compounds emitted by generalist-pollinated plants with those of specialist-pollinated plants that use private communication channels with their specialist pollinators. Some works have observed that deceptive species present a higher variability in

traits associated with pollinator attraction, including floral scent, than rewarding species (Ackerman, 2000; Salzmann *et al.*, 2007; reviewed by Juillet & Scopece, 2010).

The interaction between pollinators and herbivores constitutes an interesting line of research. The changes in floral emissions induced by systemic responses to herbivorous attacks and the implications for behavioural responses in the visitors of flowers clearly warrant investigation. Electroantennographic detection coupled with gas chromatography and mass spectrometry (GC–MS/EAD) allows the identification of volatiles from a sample while testing the recognition of its compounds by pollinators (e.g. Balao *et al.*, 2011; Gögler *et al.*, 2011; Brodmann *et al.*, 2012). Once the compounds that stimulate insect sensory system are identified, the response they elicit over insects can be tested by using behavioural assays (Chittka & Thomson, 2001). The attractive or deterrent effect of BVOCs can be verified by developing preference tests with the help of diverse olfactometer systems. The stimulus of proboscis extension reflex (PER), associated with the motivation of nectar consumption, is another response from insects to floral BVOCs that can be tested by exposing them to individual volatiles or particular blends and recording the responses they elicit (e.g. Honda *et al.*, 1998; Reinhard *et al.*, 2010; Giurfa & Sandoz, 2012).

Little evidence supports the negative selective pressure that pollinators may exert on HIPVs. Future experiments may include measurements of plant fitness for comparing plants with and without induction of HIPVs. New studies may address the effects of HIPVs on the community through their interference of plant–pollinator interactions.

Parachnowitsch *et al.* (2012) have provided an interesting work on phenotypic selection on floral scent. They demonstrate that selection towards higher floral emissions can be stronger than selection acting on other floral traits also related with pollinator attraction, such as flower colour and size. However, they did not make measurements to identify the agents of selection acting in their system and the specific importance of each one. Diverse selection agents act on flower traits; many of them are mutualists while others are antagonists and usually exert opposed selection pressures (Figure 2). In their recent work, Bartowska & Johnston (2012) have provided evidence in favour of a higher selection pressure exerted by pollinators on floral traits than the selection exerted by herbivores, although they do not include floral scent among the floral traits they consider in the work. New experiments should try to reveal the agents driving floral scent selection and the relative intensity of the selection pressures they exert.

The use of native genotypes in studies trying to understand the function of single floral volatiles or other floral traits limits the conclusions that can be obtained from these experiments, because several, frequently unmeasured, traits differ among individuals. The use of genetically transformed plants with RNAi constructs silencing the expression of many genes to avoid traits that add noise and confusion to the trait that is the focus of the study becomes a strong tool for researchers that deal with natural variation in floral scents. Plant phenotypes can be silenced to target the single expression of one volatile compound that constitutes the object of the study, and this might confer an experimental advantage to these experiments.

The drivers of global change can act on at least two levels in the role of BVOCs in plant–pollinator communication. Firstly, by affecting floral status and emissions, and secondly, by affecting the properties of BVOCs once they are released into the air. These two levels can synergistically, additively, or antagonistically affect the signals in floral scents for pollinators. These effects of global change need urgent research since a reduction in the capacity of pollinators to find flowers would have serious consequences on plant communities and agriculture. Temperature effect on flower emissions needs to be investigated. If floral emission

is a non-controlled process (Bergougnoux *et al.*, 2007) then floral emissions may be regulated only by tissue internal pools of BVOCs and by compound specific volatilities. Internal pools of BVOCs are regulated by biosynthesis processes that are temperature dependent because enzymatic activity increases with temperature. At this point only limiting availability of the biosynthetic precursor may limit this increasing effect of temperature over biosynthesis of BVOCs. On the other hand, volatility is also positively related with temperature. Temperature-volatility curves may be compound-specific; then, the ratios of emitted compounds may vary with temperature. Floral emission profiles and rates at different temperatures need to be measured to find out how temperature can affect floral signal size and composition.

Plant exposition to ozone can also change floral BVOC emissions from flowers by causing damage to the plant and eliciting the emission of stress-induced volatiles. Ozone effects over emitted flower blends must be investigated, and the consequent effects over flower visitor's behaviour need to be elucidated. Ozone is expected to react with volatiles coming from flowers and reduce their lifetime, while leading to the formation of new products. It might be interesting to investigate the potential of ozone to change floral odours perceived by pollinators and affect their capacity to find and identify flowers. This can be achieved by using reaction chambers to expose volatiles coming from flowers to ozone and by capturing the resulting mixture of volatiles to analyse them with GC–MS and more dynamically with PTR-MS-TOFF, and also to apply them on behavioural tests against non ozone-exposed floral emissions and against control air free of VOCs.

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Chapter 2. Pollination mode determines floral scent

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Abstract

The emission of floral scents is one of the many channels that plants use to communicate with pollinators. Assuming that pollinator attraction is the main function of floral volatile organic compounds (VOCs), we may thus expect them to be more abundantly emitted and more diversified in plants with biotic pollination than in those with abiotic pollination. Our main objective in this study is to determine if the pollination vector influences the potential floral emissions of flowering plants. We hypothesize that flowers pollinated by insects would emit significantly higher amounts of VOCs and would present a higher diversity of these compounds than flowers pollinated by wind. The floral emissions of 26 Mediterranean plant species were captured by dynamic headspace sampling under field conditions and analyzed by gas chromatography-mass spectrometry. Eleven species were anemophilous and fifteen were entomophilous. We searched for differences in the emission profiles between anemophilous and entomophilous flowers by considering the effects of phylogeny in our analysis. The floral emissions from the two groups were significantly different. Entomophilous species presented highly diverse emissions in both magnitude of emission rates and richness of compounds depending on the species, but overall, the flowers from entomophilous species had much higher VOC emission rates and VOC richness, both for terpenes and benzenoid compounds, than those from anemophilous species (two orders of magnitude higher emissions). The high variability in the emission rates presented by entomophilous species can result from differences in the relative reliance on visual and olfactory cues among the different species, and on the reliance on different pollinator groups with different olfactory preferences. The data thus confirm that the presence of intensely scented flowers with complex scents is strongly related to biotic pollination.

Keywords: entomophily, anemophily, floral emissions, floral scent, VOC richness.

Introduction

Anemophilous plants entrust their pollen to the wind, which serves to deliver the pollen to the stigma and fertilize the ovules. Anemophily requires a large investment in the production of male flowers with abundant pollen to ensure the pollination of few female flowers (Friedman & Barrett, 2009). On the other hand, entomophilous plants rely on visiting insects to perform their pollination. These plants have lower investments in male flowers and pollen, but they generally have higher investments in the production of rewards and signals for attracting pollinators (Friedman & Barrett, 2009). The most common floral rewards are nectar and pollen (Simpson & Neff, 1981), but some species offer oils and other less common nutritive resources to the pollinators (Bittrich & Amaral, 1997; Steiner *et al.*, 2011; Capellari *et al.*, 2012). Floral signals from entomophilous flowers serve to attract the attention of pollinators. These signals can be visual, such as a perianth with brightly-colored pigmentation (Chwil & Weryszko-Chmielewska, 2009), or olfactory, such as the strong scents of flowers (Parachnowitsch *et al.*, 2012). Pollinators can learn the floral odor of species that offer rewards and establish an association between the stimulus and the presence of these rewards (Riffell, 2011). Generalist pollinators use these specific blends of volatiles to find the flowers with the best rewards in the community, while specialist pollinators use them to find their host plants (Burger *et al.*, 2010; Filella *et al.*, 2011).

The reliance of plants on animal pollination has become a major driver of plant speciation and diversification of floral traits (Bronstein *et al.*, 2006; Whitney & Glover, 2007; Kay & Sargent, 2009). Floral scents are considered to have evolved as attractants of pollinators and have diversified extensively with biotic pollination (Whitehead & Peakall, 2009; Schiestl,

2010). Floral bouquets of volatile compounds have been found to be under strong natural selection by pollinators (Parachnowitsch *et al.*, 2012). The ability of plants to emit volatile organic compounds (VOCs) emerged early in the evolution of the plant kingdom (Kamenarska *et al.*, 2002; Fink *et al.*, 2006; Chen *et al.*, 2011). Floral structures may have emitted VOCs before the need to attract pollinators appeared in angiosperms. Floral volatiles perform functions other than attraction, the most important of which is defense, which has the opposite effect on visitors to flowers (Kessler *et al.*, 2008; Junker & Blüthgen, 2010; Schiestl, 2010; Galen *et al.*, 2011). The attractive function of floral VOCs may have effectively appeared as a modification of pre-existent VOC emissions, such as defensive terpenes that deter detrimental organisms or emissions that protect plants against stressful environmental conditions (Pellmyr & Thien, 1986). These pre-existent VOCs may have helped pollinators to identify and locate flowers that were profitable foraging resources. The display of VOCs emitted by plants has coevolved with the sensory system of pollinators resulting in new species-specific floral bouquets directed to attract particular insect species (Farré-Armengol *et al.*, 2013).

Floral VOCs are most often emitted from the perianth (corolla and calyx, containing petals and sepals, respectively) and normally in lesser amounts from other structures such as anthers or stigmas (Dobson *et al.*, 1990; Bergström *et al.*, 1995; Dötterl & Jürgens, 2005). Anemophilous flowers have diverse aerodynamic requirements for the successful liberation of pollen from anthers and its capture by stigmas that exert selective pressures on floral and inflorescence architecture. These pressures have reduced the presence of structures that can become obstacles to pollen transfer (Friedman & Barrett, 2009). From this viewpoint, anemophilous flowers, which are characterized by small petals and sepals or the lack of a perianth entirely (Ackerman, 2000; Culley *et al.*, 2002), may be expected to emit lesser amounts of VOCs per flower. The smaller size of flowers, added to the absence of a need for communication with the pollinator in anemophilous plants, leads to our hypothesis: attractive VOCs are less diversified and less abundantly emitted in anemophilous than in entomophilous flowers. In contrast, anemophilous flowers can be assumed to emit constitutive or even induced deterrent compounds directed to protect floral tissues and pollen without incurring any harmful effects on their fitness derived from the deterrence of pollinators (Dobson & Bergström, 2000).

Magalhães et al. (2005) and Wragg & Johnson (2011), measuring and comparing the emission of volatiles from flowers of diverse species from the same genus or family, found that the presence and abundance of floral volatiles depended mainly on the mode of pollination, i.e. by insects (entomophily) or by wind (anemophily). Raguso et al. (2007) found that emissions from flowers of different species of *Oenothera* were higher when flowers are pollinated by insects than by self-pollination. Doubleday *et al.* (2013) demonstrated that floral fragrance is dramatically lower in selfing compared to outcrossing populations of the species *Abronia umbellata*. Here we largely increase the range of species studied and analyze the floral emissions of several entomophilous and anemophilous Mediterranean species from diverse families to test our hypothesis while considering the effect of the phylogeny and thus to determine if the two modes of pollination have significant quantitative and qualitative differences in floral emissions. We thus aim to determine the importance of the mode of pollination on the amount and display of volatiles emitted by flowers.

Materials and methods

Scent sampling

Scent samples were captured by dynamic headspace sampling (Stashenko & Martínez, 2008) under field conditions. Flowers or inflorescences were enclosed in an oven bag (Nalophan, 20cm × 30cm), without separation from the plant. Air filtered through activated carbon was pumped into the bag via a Teflon entrance tube. Another Teflon tube collected the air exiting the bag, with one side of a T-tube connected to an adsorbent tube that collected the VOCs, followed by a flowmeter and a pump. The flux of air into the bag was always higher than the flux through the adsorbent tube to ensure that all the air from which we sampled the VOCs came from the bag. The influx was between 800 and 2000 mL min^{-1}, and the flow through the adsorbent tube was between 400 and 800 mL min^{-1}. The other side of the T-tube was open to release the excess air that did not pass through the adsorbent tube. Adsorbent tubes were filled with 114.6 mg of Tenax and 236.8 mg of Carbotrap adsorbents. Floral VOC samples were collected for five minutes. Blank samples with empty bags were collected to confirm the presence or absence of contaminating VOCs in the surrounding air and the sampling system. The filter of activated carbon used to clean the air introduced into the sampling bags did not generate air completely free of VOCs, so we collected additional controls to differentiate the environmental VOCs from those emitted by the samples. We also analyzed the air from clean unused tubes to identify possible contaminating compounds from the decomposition of tube adsorbents during thermal desorption or other contaminants from the system (Vercammen *et al.*, 2000). When the sampling of floral scents was completed, flowers of each sample were cut and dried to obtain the dry weights of the emission sources and to calculate the emission rates relative to dry weight.

The samples were collected during 2012 from different locations in central Catalonia. The criteria used to choose the species were basically two. First, we decided to select species that belong to different plant families to have a more diverse and representative sample for each pollination mode. Second, we chose species that flowered successively along the year. Five samples and one or more blank controls were collected for each species. Replicates of each species were taken on different individual plants on the same day and location. Sampling was conducted under field conditions on sunny days. Eleven anemophilous species were sampled: *Acer negundo* L., *Alnus glutinosa* L., *Coriaria myrtifolia* L., *Corylus avellana* L., *Fraxinus angustifolia* Vahl, *Olea europaea* L., *Pistacia lentiscus* L., *Populus nigra* L., *Quercus pubescens* Willd., *Ulmus minor* Mill., and *Vitis vinifera* L. Fifteen entomophilous species were sampled: *Calendula arvensis* L., *Diplotaxis erucoides* DC., *Euphorbia characias* L., *Helichrysum stoechas* L., *Lepidium draba* L., *Ligustrum japonicum* Thunb *Prunus dulcis* Mill., *Rhamnus alaternus* L., *Salvia verbenaca* L., *Sambucus nigra* L., *Syringa vulgaris* L., *Thymus vulgaris* L., *Tilia platyphyllos* Scop., *V. lantana* L., and *Viburnum tinus* L.

Scent analyses

VOC analyses were performed by gas chromatography-mass spectrometry (Agilent Technologies, GC: 7890A, MS: 5975C inert MSD with Triple-Axis Detector, Palo Alto, CA, USA). The adsorbent tubes were thermally desorbed, and samples were injected into a 30 m x 0.25 mm capillary column with a 0.25 µm film thickness (HP-5MS, Agilent Technologies). Helium flow was 1 mL min^{-1}. Total run time was 26 min. After sample injection, the initial time was 1 min, and the initial temperature (35°C) was increased at 15°C.min^{-1} to 150°C and maintained for 5 min, then at 50°C.min^{-1} to 250°C and maintained for 5 min and finally at 30°C.min^{-1} to 280°C and maintained for 5 min.

VOCs were identified by comparing the retention times with liquid standards from Fluka (Buchs, Switzerland) injected into clean adsorbent tubes, and the fractionation mass spectra were compared with standard spectra and the Nist05a and wiley7n mass spectra libraries. VOC concentrations were determined from calibration curves. The calibration curves for the common VOCs α-pinene, β-pinene, limonene, α-humulene, 3-hexen-1-ol and dodecane were determined once every seven analyses. VOC calibration curves (n=4 different terpene concentrations) were always highly significant (r^2>0.99) for the relationship between signal and VOC emission rates. We calculated the emission rates of VOCs relative to the dry weights of the flowers ($\mu g.gDW^{-1}.h^{-1}$), subtracting the emission rates of the blanks from their respective flower samples.

Statistical and phylogenetic analyses

We conducted PERMANOVA analyses of the floral VOC emission rates with R software. PERMANOVA is a permutational multivariate analysis of variance for testing the simultaneous response of multiple variables to one or more factors on the basis of any distance measure using permutation methods (Anderson, 2006). For this purpose we used the function *adonis* from the package *vegan* (Oksanen *et al.*, 2013). Since the data on the emission rates and richness of terpenoids, benzenoids and total VOCs did not show normal distribution we used the Kruskal-Wallis non parametric test for the comparison of anemophilous and entomophilous floral emissions.

We constructed a phylogenetic tree and obtained the phylogenetic distances among species with Phylomatic and Phylocom (Webb & Donoghue, 2005; Webb *et al.*, 2008). Briefly, Phylomatic uses a backbone plant megatree based primarily on DNA data from a variety of 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 (Webb & Donoghue, 2005). We used the package *picante* from R software to test for phylogenetic signals in the floral emissions of the species studied. The function *phylosignal* calculates a statistic of phylogenetic signal (Blomberg's K) as well as a *P*-value based on variance of phylogenetically independent contrasts relative to tip shuffling randomization (Blomberg *et al.*, 2003). We used the package *ape* from R software to read and plot the phylogenetic tree and PermutMatrix (Caraux and Pinloche 2005) to construct the image map.

Results

The emission profiles of entomophilous samples were highly diverse (Figures 1-3), with different magnitudes of VOC emission rates (Figures 1-3) and different levels of VOC diversity (Figures 1 and 3, Table 1). The majority of anemophilous species had low or null floral emission rates, compared with entomophilous species (Figure 3).

Table 1. Pollination mode and floral emission rates (μg g DW^{-1} h^{-1}) of single and total VOCs for each species. References supporting the pollination mode of each species are provided: [1]Fernández-Rodríguez et al. (2013), [2]Thompson & Gornall (1995), [3]Cuevas & Polito (2004), [4]Verdú & García-Fayos (1998), [5]Herrera (1987), [6]Imbert & Lefèvre (2003), [7]Fernández-Martínez et al. (2012), [8]López-almansa et al. (2004), [9]Di Vecchi-Staraz et al. (2009), [10]Orueta (2002), [11]Kunin (1992), [12]Blancafort & Gómez (2005), [13]Scurfield (1962), [14]Honda et al. (1998), [15]Gradziel (2009), [16]Aronne & Wilcock (1995), [17]Navarro (1997), [18]Atkinson & Atkinson (2002), [19]Denisow & Strzałkowska-Abramek (2014), [20]Matesanz et al. (2911), [21]Hesse (1993), [22]Kollmann & Grubb (2002), [23]Nebot & Mateu (1990). Asterisks (*) indicate field observations of flower-visiting insects conducting visits to these species.

	Pollination mode	Floral emission rates of single VOCs	Total floral VOC emission rates
Acer negundo	Anemophily[1]	(E)-β-ocimene (0.057±0.03), (Z)-3-hexen-1-ol (12±7.7), tetradecane (1.1±1.01), dodecanoic acid (0.16±0.05), hexadecane (1.13±0.94), tetradecanoic acid (1.20±0.23), pentadecanoic acid (0.54±0.13), n-hexadecanoic acid (3.19±0.95)	19.4±10.4
Alnus glutinosa	Anemophily[1]	3-carene (0.036±0.024), 1R-α-pinene (0.18±0.13), (E)-β-ocimene (0.023±0.013), D-limonene (0.6±0.54), camphene (0.041±0.031), β-phellandrene (0.002±0.002), benzenecarboxylic acid (0.98±0.24), dodecane (0.37±0.16), decanal (0.31±0.13), nonanoic acid (0.58±0.23), tridecane (0.068±0.029), tetradecane (0.34±0.13), pentadecane (0.42±0.19), hexadecane (0.37±0.2)	4.3±1.6
Coriaria myrtifolia	Anemophily[2]	ethylbenzene (0.28±0.1), p-xylene (1.07±0.43), o-xylene (0.57±0.25), decane (0.23±0.13),1,3,5-trimethyl-benzene (0.5±0.3), undecane (0.26±0.19), nonanal (0.61±0.38), dodecane (0.4±0.22), decanal (0.63±0.35), tetradecane (0.54±0.32), tetradecanoic acid (1.01±0.21), pentadecanoic acid (0.64±0.15), n-hexadecanoic acid (4.2±0.95), octadecanoic acid (0.6±0.22)	11.5±3.2
Corylus avellana	Anemophily[1]	3-carene (0.014±0.01), 1R-α-pinene (1.05±1.03), camphene (0.12±0.07), benzenecarboxylic acid (0.88±0.65), hexanal (0.18±0.11), heptanal (0.23±0.08), nonanoic acid (0.76±0.41), n-decanoic acid (0.412559), tetradecane (0.71±0.63), dodecanoic acid (0.068±0.031)	4.4±2.3

	Pollination mode	Floral emission rates of single VOCs	Total floral VOC emission rates
Fraxinus angustifolia	Anemophily[1]	3-carene (0.27±0.17), 1R-α-pinene (2.88±2.21), D-limonene (2.64±1.66), camphene (0.23±0.16), ethylbenzene (0.59±0.48), p-xylene (1.99±1.84), benzenecarboxylic acid (0.26±0.08), nonanal (0.38±0.32), dodecanoic acid (0.49±0.14), tetradecanoic acid (0.24±0.12), pentadecanoic acid (0.21±0.08), n-hexadecanoic acid (0.72±0.4), eicosane (0.08±0.04)	11.0±7.1
Olea europaea	Anemophily[3]	tetradecane (0.76±0.64), pentadecanoic acid (0.14±0.12), n-hexadecanoic acid (0.86±0.75)	1.8±1.5
Pistacia lentiscus	Anemophily[4, 5]	3-carene (0.13±0.04), 1R-α-pinene (0.47±0.28), β-pinene (0.12±0.07), (E)-β-ocimene (0.12±0.05), D-limonene (0.32±0.23), α-phellandrene (0.74±0.49), β-phellandrene (0.33±0.24), dodecanoic acid (0.16±0.08), pentadecanoic acid (0.06±0.04), n-hexadecanoic acid (0.52±0.27), E-9-octadecenoic acid (0.09±0.047), octadecanoic acid (0.19±0.09)	3.2±1.5
Populus nigra	Anemophily[6]	not detected	not detected
Quercus pubescens	Anemophily[7]	not detected	not detected
Ulmus minor	Anemophily[8]	3-carene (0.24±0.21), 1R-α-pinene (1.34±0.74), D-limonene (0.28±0.25), camphene (0.11±0.06), decane (0.047±0.019), 1,2,3-trimethyl-benzene (0.066±0.046), undecane (0.091±0.014), decanal (0.068±0.038), hexadecane (0.064±0.042), octadecane (0.027±0.018)	2.3±0.9
Vitis vinifera	Anemophily[9]	not detected	not detected

	Pollination mode	Floral emission rates of single VOCs	Total floral VOC emission rates
Calendula arvensis	Entomophily[10]	3-carene (98±29), 1R-α-pinene (728±80), β-pinene (99±20), D-limonene (230±24), camphene (216±62), α-phellandrene (64±19), β-phellandrene (64±12), α-terpinene (121±77), γ-terpinene (87±20), β-myrcene (115±25), sabinene (707±91), α-cubebene (20±2.8), (Z)-3-hexen-1-ol acetate (37±24), 1,2,3,4-tetramethyl-benzene (420±155),1-methyl-4-(1methylethenyl)-benzene(233±62), nonanal (38±2.5), dodecane (24±14), nonanoic acid (5.5±2.3), tridecane (15.5±4.4), tetradecane (31±15), dodecanoic acid (5.5±2.2), hexadecane (21±5.3), tetradecanoic acid (8.5±5.4), pentadecanoic acid (5.6±4.3), n-hexadecanoic acid (36±26), E-9-octadecenoic acid (4.7±3), octadecanoic acid (13±7.4)	3447.9±553.4
Diplotaxis erucoides	Entomophily[11,] *	3-carene (1.2±0.48), 1R-α-pinene (16±4.6), β-pinene (1.16±0.32), α-ocimene (1.4±0.49), (E)-β-ocimene (1.15±0.45), D-limonene (24±7.7), camphene (0.66±0.23), α-phellandrene (1.05±0.3), β-myrcene (1.73±0.44), acetic acid (4.2±2.1), octane (0.7±0.37), ethylbenzene (2.9±1.11), p-xylene (8.1±4.2), o-xylene (3.4±1.8), benzaldehyde (1.36±0.83), 1,3,5-trimethyl-benzene (2.15±1.02),1-ethyl-2-methyl-benzene (2.2±1), heptanal (3.3±0.9), dodecane (4.1±1.5), decanal (1.92±1.18), tridecane (3.2±1.3), tetradecane (4. 1±1.9), hexadecane (6.6±4.5), tetradecanoic acid (24±11), pentadecanoic acid (6.3±3), Z-11-hexadecenoic acid (5±3.1), n-hexadecanoic acid (63±30), octadecanoic acid (16±9.2)	210.8±55.3
Euphorbia characias	Entomophily[12]	camphene (0.42±0.42), β-phellandrene (0.66±0.61), benzaldehyde (3.4±2.9), hexanal (0.73±0.31), octadecanoic acid (1.47±0.25)	6.7±3.6

	Pollination mode	Floral emission rates of single VOCs	Total floral VOC emission rates
Helichrysum stoechas	Entomophily[5,] *	1R-α-pinene (14.3±2.8), 1S-α-pinene (0.56±0.13), camphene (1.78±0.4)	16.6±3.1
Lepidium draba	Entomophily[13,]*	1R-α-pinene (0.028±0.012), (E)-β-ocimene (0.033±0.014), D-limonene (0.18±0.05), γ-terpinene (0.039±0.014), benzaldehyde (0.18±0.04), benzenecarboxylic acid (0.72±0.08), undecane (0.068±0.018), dodecane (0.14±0.03), tridecane (0.17±0.06), tetradecane (0.23±0.08), pentadecanoic acid (0.37±0.09), n-hexadecanoic acid (3.1±1.2), E-9-octadecenoic acid (0.69±0.31), octadecanoic acid (1.88±1.32)	7.9±3.1
Ligustrum japonicum	Entomophily[14,]*	(E)-β-ocimene (14.5±1.9), (Z)-β-ocimene (0.57±0.05), benzaldehyde (1.91±0.57)	17.0±2.5
Prunus dulcis	Entomophily[15,]*	1R-α-pinene (8.7±5.1), β-pinene (0.43±0.25), (E)-β-ocimene (1±0.17), D-limonene (5.7±4.3), camphene (0.23±0.23), α-phellandrene (0.6±0.4), β-phellandrene (0.25±0.11), β-myrcene (0.54±0.3)	17.4±10.5
Rhamnus alaternus	Entomophily[16,]*	3-carene (0.66±0.37), 1,2,3-trimethyl-benzene (0.26±0.13), tetradecane (1.07±0.84)	2.0±1.3
Salvia verbenaca	Entomophily[17]	1R-α-pinene (1.19±0.53), α-ocimene (1.42±0.35), (E)-β-ocimene (2.54±0.77), D-limonene (2.27±0.57), β-phellandrene (1.23±0.55), β-myrcene (0.43±0.31), α-fenchene (0.43±0.09), lilac aldehyde A (2.9±1), lilac aldehyde C (2.41±0.73), p-xylene (122±62), 1,2,4-trimethyl-benzene (134±89)	270.9±123.4
Sambucus nigra	Entomophily[18,]*	3-carene (2.94±0.79), (E)-β-ocimene (1.27±0.15), linalool oxide (0.42±0.08), (Z)-3-hexen-1-ol (1.15±0.3), p-xylene (0.097±0.037), benzenecarboxylic acid (2.03±0.72)	7.9±1.6

	Pollination mode	Floral emission rates of single VOCs	Total floral VOC emission rates
Syringa vulgaris	Entomophily[19,]*	3-carene (0.3±0.19), 1Rα-pinene (0.32±0.17), β-pinene (0.098±0.054), (E)-β-ocimene (1.01±0.47), (Z)-β-ocimene (0.042±0.021), D-limonene (0.025±0.015), lilac aldehyde B (0.84±0.63), lilac aldehyde C (0.6±0.45), p-xylene (0.047±0.019),(methoxymethyl)-benzene (0.069±0.031), benzeneacetaldehyde (0.94±0.43), 1,2,4-trimethyl-benzene (0.023±0.013), benzenecarboxylic acid (0.28±0.25), 1,4-dimethoxy-benzene (1.43±1.09), nonanoic acid (0.23±0.17), dodecanal (0.0.38±0.025), pentadecane (0.031±0.01), hexadecane (0.059±0.019), nonandecane (0.024±0.014), 1,2-benzenedicarboxylic acid (0.068±0.02)	6.5±3.9
Thymus vulgaris	Entomophily[20,]*	3-carene (25±5), 1R-α-pinene (21±5.4), β-pinene (5.7±1.5), (E)-β-ocimene (1.98±0.91), D-limonene (22±8.8), eucalyptol (5.8±1.7), borneol (22±8.6), caryophyllene (10.9±4.8), camphene (28±7.2), camphor (4.3±1.7), thymol (10.1±3.9), α-phellandrene (14.1±5.3), α-terpinene (9.8±1.8), γ- terpinene (214±50), β-myrcene (44±13), sabinene (4.2±1.1), (Z)-3-hexen-1-ol acetate (5.9±3.1), benzaldehyde (2.45±1.32), E-9-octadecenoic acid (4.1±2.2), octadecanoic acid (18±7.8)	472.2±90.6
Tilia platiphyllos	Entomophily[21,]*	(E)-β-ocimene (0.39±0.05), α-terpinolene (0.096±0.012), (E)-9-octadecenoic acid (0.12±0.08), octadecanoic acid (0.17±0.10)	0.8±0.2
Viburnum lantana	Entomophily[22,]*	linalool (1.27±0.38), β-myrcene (0.21±0.07), lilac aldehyde A (0.14±0.02), lilac aldehyde B (0.18±0.02), lilac aldehyde D (0.045±0.004), dodecane (0.04±0.02), tridecane (0.047±0.019), tetradecane (0.15±0.08), hexadecane (0.088±0.042)	2.2±0.4
Viburnum tinus	Entomophily[23]	not detected	not detected

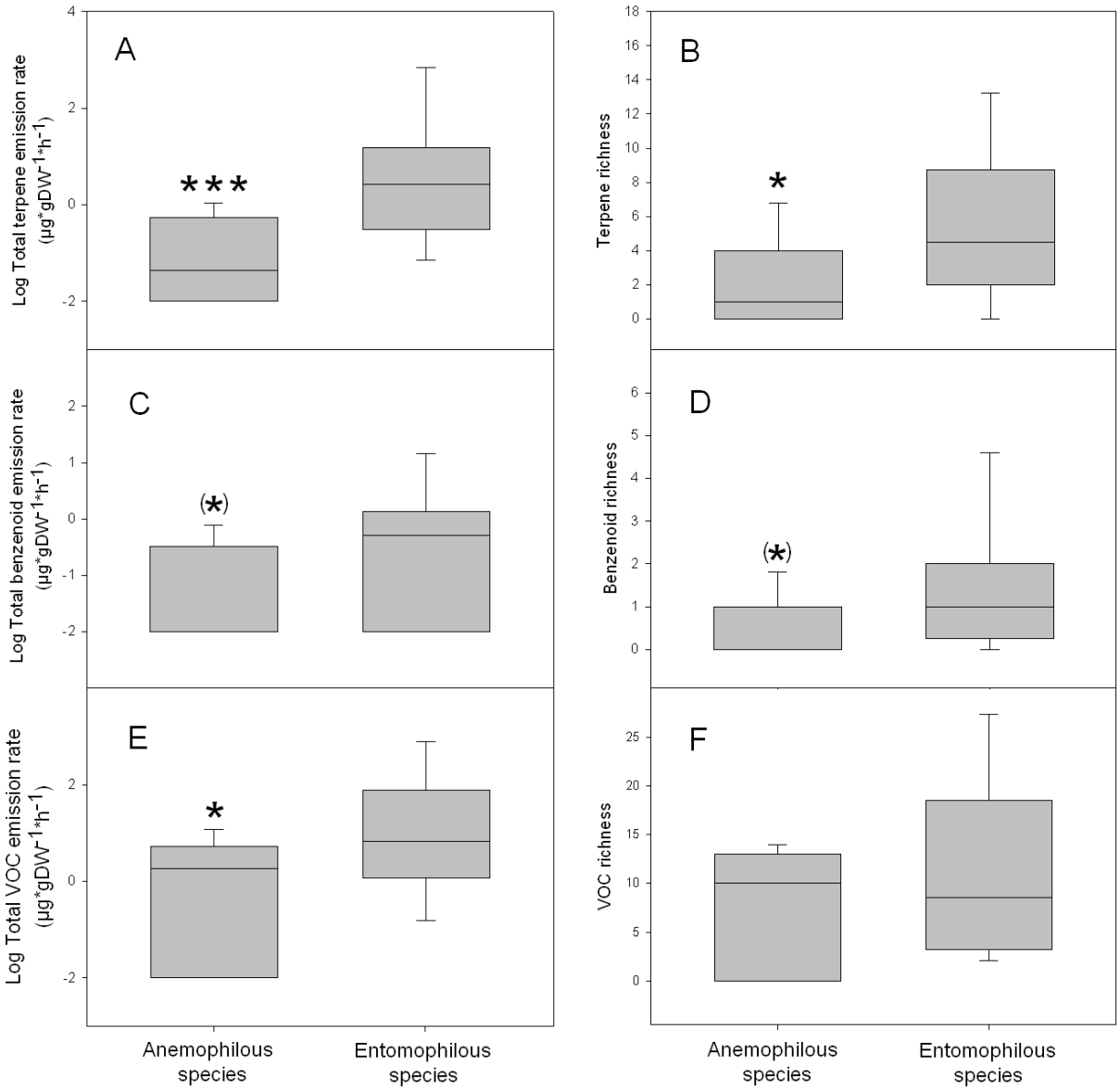

Figure 1. Box plots of A) logarithm of total terpene emission rates, B) terpene richness; C) logarithm of total benzenoid emission rates, D) benzenoid richness; E) logarithm of total VOC emission rates, and F) VOC richness, for anemophilous (n=11) and entomophilous (n=15) species. Asterisks indicate the level of significance: $^{(*)}$ (P<0.1), * (P<0.05), ** (P<0.01), *** (P<0.001).

When focusing on terpenes, we detected that entomophilous species had higher mean and higher variance in their total terpene emission rates than anemophilous species (194.8± 159.3and 1.1± 0.6 µg.gDW^{-1}.h^{-1}, respectively; Kruskal-Wallis test, χ^2=6.8, P=0.009) and higher mean terpene richness (5.6± 1.1 and 2.3± 0.8 compounds, respectively; χ^2=3.57, P=0.06) (Figure 1). Total terpene emission rates showed phylogenetic signal (K=0.68, P=0.05, Table 2).

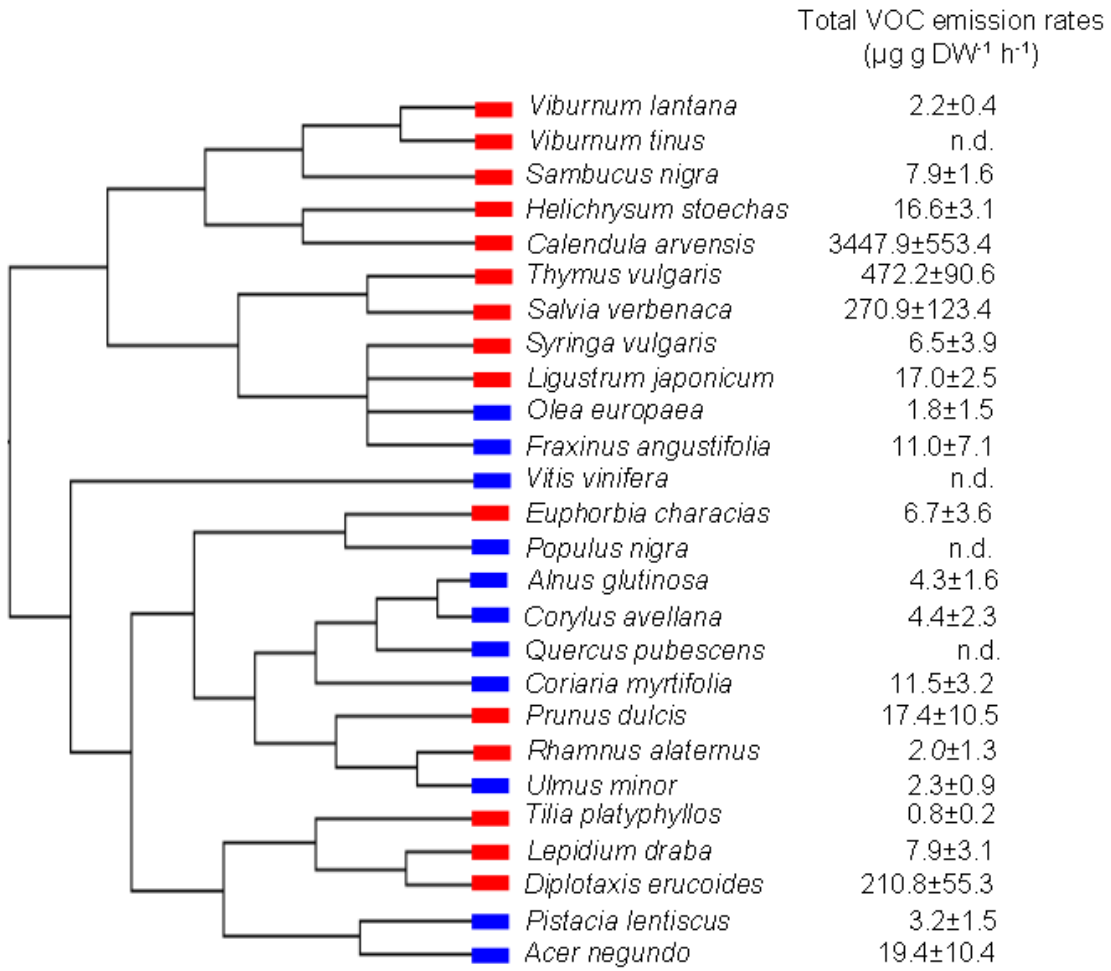

Figure 2. Phylogenetic tree with the phylogenetic distances among the species sampled in this study. The modes of pollination of each species are identified by blue (anemophilous) and red (entomophilous) squares. Total VOC emission rates (µg g DW^{-1} h^{-1}) are provided for each species.

Table 2. Results of the phylogenetic signal tests for total terpene emission rates, total benzenoid emission rates, total VOC emission rates, terpene richness, benzenoid richness and VOC richness. Bloomberg's K and *P*-values are provided for each variable.

	K	*P*
Total terpene emission rates	0.68	0.05
Total benzenoid emission rates	0.68	0.07
Total VOC emission rates	0.69	0.06
Terpene richness	0.54	0.14
Benzenoid richness	0.46	0.45
VOC richness	0.47	0.35

With respect to benzenoids, our results show that entomophilous species had higher mean and higher variance in their total benzenoid emission rates than anemophilous species (50.6± 41 and 0.3± 0.1 µg.gDW^{-1}.h^{-1}, respectively; Kruskal-Wallis test, χ^2=4.1, *P*=0.04) and higher mean benzenoid richness (1.4± 0.4 and 0.5± 0.2 compounds, respectively; χ^2=2.93, *P*=0.09) (Figure 1). Total benzenoid emission rates showed phylogenetic signal that was close to significance (K=0.68, *P*=0.07, Table 2).

Entomophilous species had higher mean and higher variance in their total VOC emission rates than anemophilous species (280.5 ± 213.8 and 5.3± 1.9 µg.gDW^{-1}.h^{-1}, respectively; Kruskal-Wallis test, χ^2=3.5, *P*=0.06) and also a not significant higher VOC richness (10.7 ± 2.2 and 7.6 ± 1.7 compounds, respectively; χ^2=0.39, *P*=0.53) (Figure 1). The PERMANOVA analyses using all single compound emission rates showed that the floral emission rates of anemophilous and entomophilous species differed significantly (pseudo-F=3.11, *P*=0.02). The phylogenetic signal of total VOC emission rates was close to significance (K=0.69, *P*=0.06, Table 2).

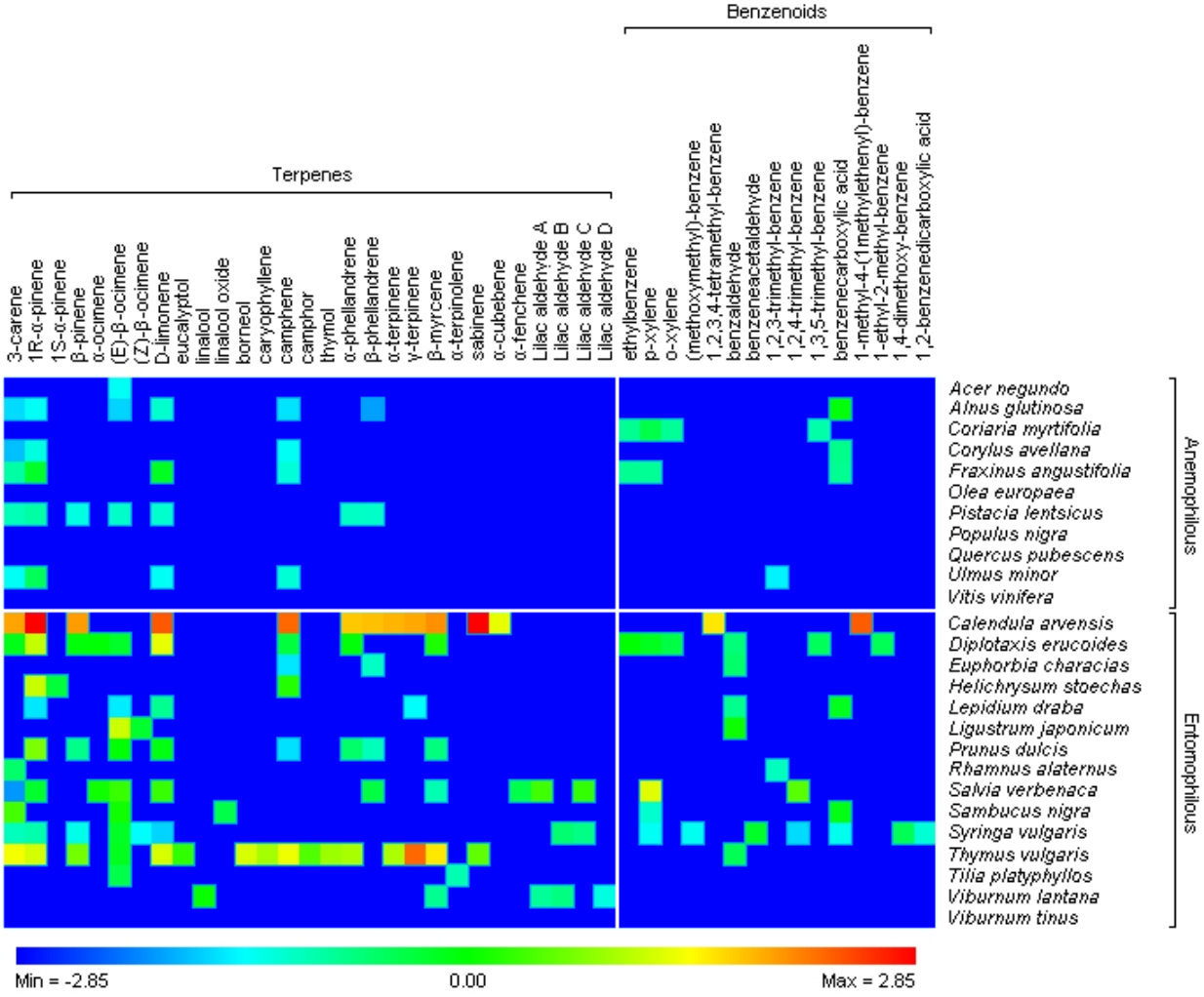

Figure 3 – Image map of terpene and benzenoid emission rates of flowers for anemophilous and entomophilous species. The data is expressed as log (µg g DW^{-1} h^{-1}).

Discussion

Our results indicate that anemophilous and entomophilous flowers have different VOC emissions. Entomophilous flowers showed considerably higher, two orders of magnitude, VOC emission rates than did anemophilous flowers (figs 1-3). The emissions of entomophilous flowers were composed of a higher diversity of compounds, especially terpenes (Figure 1). These results support our hypothesis that plants with biotic pollination have usually higher emission rates and more complex scents than do plants with abiotic pollination, although this does not always occur, which leads to the high variability presented by entomophilous species.

High emission rates were measured for *S. verbenaca* and *T. vulgaris*, two species that belong to the Lamiaceae, a family rich in aromatic species with abundant glandular cells in their vegetative and floral tissues (Ascensão et al., 1999). Another species without specialized VOC storage structures, such as *C. arvensis* of the family Asteraceae, also had high floral emission rates and a high diversity of compounds. Some entomophilous flowers, such as those of *V. tinus* and *T. platiphyllos*, had low emission rates per mass of flower. The high variability in the emission rates presented by entomophilous species can result from differences in the relative reliance on visual and olfactory cues among the different species. Some species may rely more on visual cues to attract pollinators and emit lower amounts of floral VOCs, while others emit strong floral scents. The level of reliance that flowers present on olfactory and other sensory channels may depend on the sensory abilities of the pollinators that they attract (Chittka and Raine, 2006; Fink et al., 2006; Schaefer et al., 2004). For example, *Petunia axillaris* and *P. exserta*, two closely related species with different pollination syndromes, show divergent reliability on visual and olfactive cues to attract their respective pollinators. The former, which attracts nocturnal moths, has colorless flowers that emit strong scents composed of benzenoids, while the latter attracts hummingbirds with red non-scented flowers (Klahre et al., 2011). Species from the genus *Clarkia* present non-scented flowers that are pollinated by bees, while one species, *C. breweri*, have evolved a strong floral scent composed of monoterpenes and benzenoids that attract moths to their flowers (Dudareva et al., 1996).

Entomophilous species also presented a high diversity of floral volatile compounds (Figure 3). *S. verbenaca* and *T. vulgaris*, aromatic plants of the family Lamiaceae, emitted a high diversity of floral VOCs compared with other entomophilous species. This indicates that some plant lineages can present a higher display of floral VOCs than others. Plants with a specialist pollination system may emit floral chemical messages directed to their particular range of pollinators. This can be achieved by using uncommon VOCs that are not present in the floral blends of other species as well as by emitting complex floral blends with unique combinations and proportions of more ubiquitous VOCs (Farré-Armengol et al., 2013; Raguso, 2008). The need of species with specialist pollination to produce a unique floral scent can therefore stimulate the chemical richness of their floral VOC emissions. On the other side, some flowers visited by generalist pollinators may be able to use simpler blends composed of general attractants, such as the common floral monoterpene β-ocimene (Filella et al., 2013; Knudsen et al., 2006).

The variability in the VOC composition of floral scents among entomophilous species has been described to depend on the reliance on different pollinator groups with different olfactory preferences (Dobson, 2006). For example, *L. japonicum*, *S. vulgaris* and *V. lantana* are visited by butterflies (field observations), and their floral scents are mainly composed of benzenoids, β-ocimene, linalool and lilac aldehydes (Table 1). The ubiquity of bezenoids and the monoterpene linalool in the floral scent of plants pollinated by butterflies and moths has suggested that these compounds are used as attractants of Lepidoptera (Andersson et al., 2002; Dötterl et al., 2006). The common monoterpene (E)-β-ocimene has been found to elicit strong antennal responses in the butterfly *Heliconius melpomene* (Andersson and Dobson, 2003) and lilac aldehydes are common in some nectar plants and elicit antennal responses in butterflies and moths (Andersson, 2003; Dötterl et al., 2006). The use of particular VOCs as attractants of a specific kind of pollinator by taking advantage of the innate olfactory preferences shared by insects of the same group may not stimulate higher floral VOC richness within species but may stimulate the differentiation of the floral scent composition among entomophilous species with different pollination syndromes.

Some entomophilous species are self-compatible and can naturally experience different degrees of self-pollination, which does not involve the need to provide chemical cues

to attract pollinators. For example, the central flowers of *C. arvensis* capitula are self-pollinated, while the peripheral flowers can experience crossed pollination mediated by insects (Heyn, 1988). *Calendula arvensis* plants produce bigger achenes with higher resource reserves and more pronounced appendices for dispersal by wind and animals from peripheral flowers of the capitula, thus showing that insect mediated crossed pollination is positively selected. Although *D. erucoides* plants have the capacity of being self-pollinated, they have several floral traits that stimulate pollinator attraction, such as big scented flowers grouped in dense inflorescences (Sans and Bonet, 1993). Self-pollination results in significantly lower seeds per siliqua than crossed pollination in *D.erucoides* plants, and also reduces population genetic variability and evolutionary capacity. Self-pollination can also occur in *Thymus vulgaris,* but only in hermaphrodite individuals and in very low rates (Thompson and Tarayre, 2000). *Salvia verbenaca* flowers stimulate self-pollination if outcrossing pollen has not been received during the first floral stages, but crossed pollination stimulates higher seed weight and higher reproductive success (Navarro, 1997). The self-compatible entomophilous species of this study showed the strongest and most diversified floral scents, thus highlighting the importance of maintaining certain levels of crossed pollination even in self-compatible entomophilous species, which can partially rely on self-pollination when biotic pollination vectors are scarce.

Our results demonstrate that the mode of pollination is a crucial factor determining the display of floral VOCs in flowering plants. We conclude that anemophilous species overall present less diversity and lower amounts of floral VOCs than do entomophilous species and that floral scents are though highly variable among entomophilous species. While a few entomophilous species emitted low amounts of VOCs, others emitted high amounts and diversities of VOCs. We argue that this variability can be the result of differences in the pollinators to which flowers direct their signals, what can involve different levels of reliance on olfactory signals against other sensory channels. The floral chemistry of some entomophilous species may also depend on third parties as selective agents through eavesdropping and fitness loss. We thus conclude that the reliance on biotic agents for pollination is a major factor determining the selection for the appearance and significance of floral VOC emissions and of the expression of their synthesis and emission in floral tissues.

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Chapter 3. Relationships among floral VOC emissions, floral rewards and visits of pollinators in five plant species of a Mediterranean shrubland

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Abstract

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

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

Introduction

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

pollinators (Farré-Armengol *et al.*, 2013), particularly the intricate relationship of signals between plants and pollinators in diverse plant-pollinator communities (Kessler & Halitschke, 2009; Vázquez *et al.*, 2009).

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

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

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

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

plant parts (Farré-Armengol *et al.*, 2013). Moreover, terpenoids have been suggested to be major contributors to the effect of floral scent emissions on seed fitness (Majetic *et al.*, 2009).

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

In a recent study, Filella *et al.* (2013) conducted a series of measurements in a Mediterranean coastal shrubland community, and found that floral volatile emissions were highest in the species flowering during the first months of spring. The flowers presented maximal rewards when pollinator visitation rates were at a minimum. Volatile emissions were lowest in those species flowering in late spring-early summer when the availability of rewards was lower and pollinator visitation rates were at a maximum. These relationships are of great interest for the resource economy of plants, which is strongly influenced by the large investment in floral resources that most plants assume during their blooming periods. A possible reduction in investment in floral signals and rewards in the final stage of the community's peak flowering period by spring- and summer-blooming species (when many pollinators have fewer floral resources available to them) can lead to a considerable saving of resources (Gershenzon, 1994) without implying a decrease in plant fitness, because pollinators are more abundant and active and fewer plant species in flower are competing for the services of pollinators. Here, we addressed this question at the intraspecific level, i.e. we aimed to determine whether floral BVOC emissions and floral rewards (pollen and nectar) decrease along the flowering period of each single species, coinciding with the described seasonal pattern of increasing abundance and activity of pollinators and decreasing numbers of coexisting plant species in flower. Our hypothesis assumes that this would be advantageous for the plant to maximize the investment in flower rewards and signals when there is maximal competition for pollinators, and reduce this investment when competition decreases to save a significant amount of resources. We tested these possible patterns in five plant species encompassing a range of flowering periods: early spring (*Rosmarinus officinalis* L. and *Muscari neglectum* Guss.), late spring (*Euphorbia flavicoma* DC. and *Phlomis lychnitis* L.), or throughout the entire spring period (*Biscutella laevigata* L.).

Materials and methods

Study area and sampling design

Field work was performed at Garraf Natural Park on the central coast of Catalonia (NE Spain) in 2011. The experimental zone was located at 400 m a.s.l. and 2800 m from the coast (UTM: 31T, 408256 m, 4570749 m). The climate is typically Mediterranean and is strongly influenced by proximity to the sea, with sparse but torrential rain during spring and autumn, temperate winters, and hot and dry summers. The plant community in the sampling zone is a shrubland dominated by Kermes oak (*Quercus coccifera* L.) and mastic tree (*Pistacia lentiscus* L.), with

dwarf fan palm (*Chamaerops humilis* L.), Mauritania vine reed (*Ampelodesmos mauritanica* (Poir.) T.Durand & Schinz), Killarney strawberry tree (*Arbutus unedo* L.), Mediterranean buckthorn (*Rhamnus alaternus* L.), rosemary (*Rosmarinus officinalis*), Mediterranean heath (*Erica multiflora* L.), Salvia cistus (*Cistus salviifolius* L.), and a large variety of geophytes (*Muscari neglectum*, *Gladiolus illyricus* W.D.J. Koch, *Ranunculus* sp. L., *Anacamptys piramidalis* (L.) Rich., and *Narcissus assoanus* Dufour ex Schult. & Schult.f.) and dwarf shrubs (*Helianthemum* sp., *Euphorbia flavicoma*, *Polygala rupestris* Pourr., *Biscutella laevigata*, and *Phlomis lychnitis*). The community of pollinators and plants present in this area and their relationships are described in detail in Bosch *et al.* (2009).

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

Pollinator visitation rate

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

Nectar and pollen production

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

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

BVOC emission rates

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

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

Monoterpenes were identified by comparing the retention times with liquid standards from Fluka (Buchs, Switzerland) volatilised in vials and the fractionation mass spectra with standard spectra and Nist05a and wiley7n mass spectra libraries. Terpene concentrations were determined from calibration curves. The calibration curves for the common monoterpenes α-pinene, β-pinene, 3-carene, and linalool and common sesquiterpenes such as α –humulene were determined once every seven analyses. Terpene calibration curves (n = 4 different

terpene concentrations) were always highly significant (*r2* > 0.99) in the relationship between signal and terpene emission rates.

Simultaneously with the GC-MS measurements, other floral samples (one inflorescence for *M. neglectum* and one or two flowers for the other four species) from the same individual plants were clamped in a 90 cm3 PLC-2 ADC cuvette connected to an infrared gas analyser (LCA-4, ADC; Hoddeson, Hertfordshire, UK). BVOC-free zero air was fluxed into the cuvette, and the exiting air was sent to a PTR-MS system. The cuvette was maintained at 30°C and 1000 µmol m^{-2} s^{-1} PAR. Because the flow through the cuvette (about 250 mL/min) was higher than the flow needed for PTR-MS (50–100mL/min), part of the flow was channelled through an overflow outlet. The cuvette was lined with Teflon, and only Teflon tubing, connectors, and valves were used, to reduce the surface interactions in the system. The measurements of gas exchange were also conducted with an empty cuvette as an additional control. Part of the air exiting the leaf cuvette thus flowed through a T-system to the PTR-MS inlet. The PTR-MS is a highly sensitive device (PTR-MS-FTD hs; Ionicon Analytik, Innsbruck, Austria) consisting of three parts: the ion source, where ions are produced by a hollow cathode discharge using water vapour as the molecular source of ions; the drift tube, where proton-transfer reactions to the trace constituents in the air occur (BVOCs with a higher proton affinity than that of water (166.5 kcal mol−1), including most unsaturated and almost all oxygenated hydrocarbons, undergo a proton-transfer reaction with H3O+); and the ion detector, which provides sensitive detection of the mass-selected ions that are characteristic of the molecules of interest. Both the PTR-MS and its use in BVOC analysis have been described in detail elsewhere (Lindinger *et al.*, 1998; Peñuelas *et al.*, 2005b). Here, the PTR-MS drift tube was operated at 2.1 mbar and 40°C, with a drift field of 600 V cm−1. The parent ion signal was maintained at ca. 2 × 106 counts s −1 during the measurements. We conducted scans of all masses between 41 and 206.

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

Statistical treatment

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

Results

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

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

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

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

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

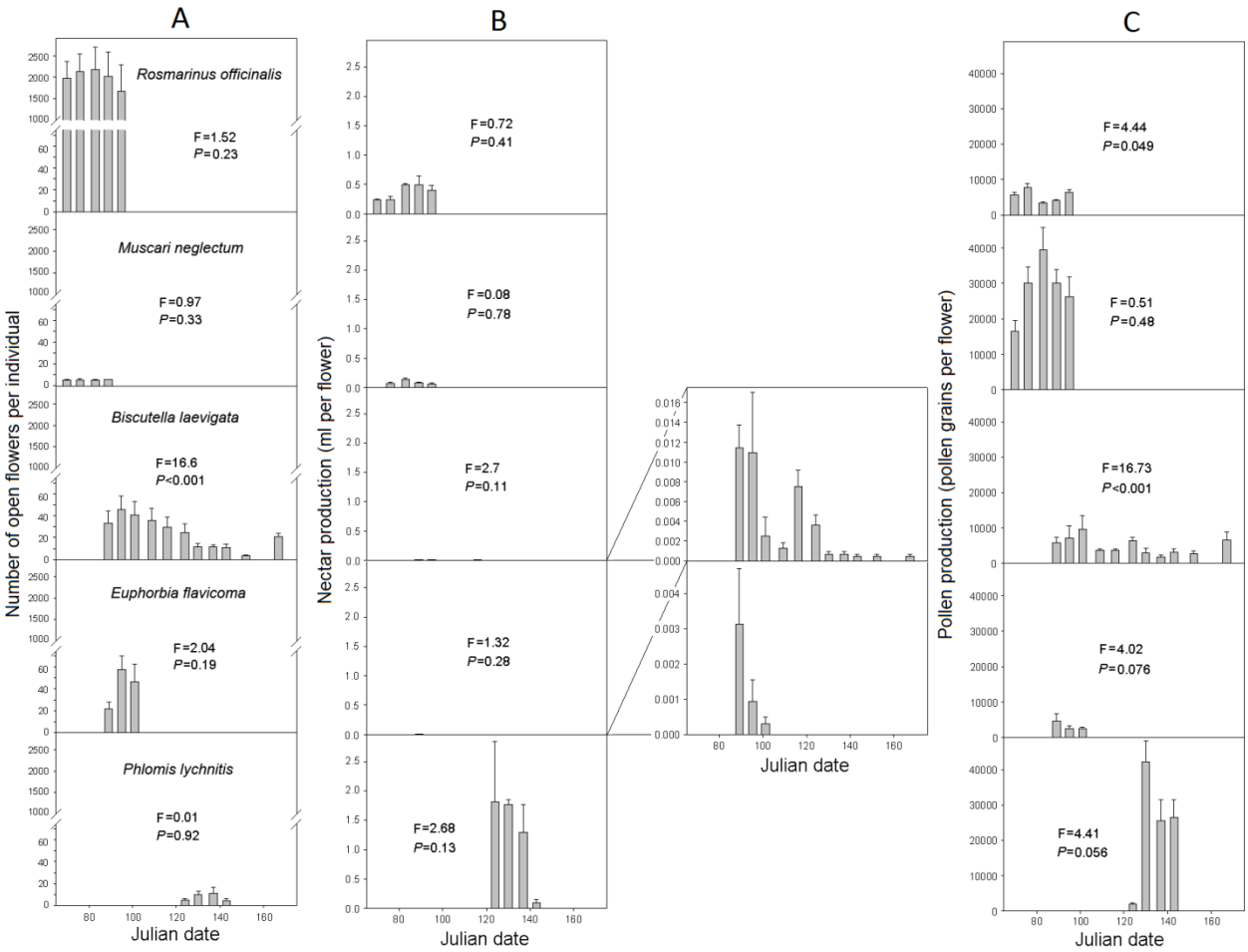


Figure 1. A, mean values of total number of flowers per individual of the five plant species during their flowering periods in year 2011; B, mean values of nectar production per flower of the five plant species during their flowering periods in year 2011; C, mean values of pollen production per flower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

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

Relation of floral rewards and volatile emissions with visitation rates

Visitation rates showed a significant negative correlation with pollen and nectar in the case of *B. laevigata* (F = 7.18, *P* = 0.01 and F = 4.64, *P* = 0.037, respectively). Floral rewards showed no significant correlations with visitation rates in the other species (*P* > 0.05). Visitation rates were found to present a significant positive correlation with terpene emission capacities in *M. neglectum* (F = 5.22, *P* = 0.03). The generalized linear mixed model conducted with pollinator visitation rates as the response variable, and with pollen and nectar production, terpene emission capacity and temperature as explanatory variables, was found significant for *B. laevigata*, and floral rewards were the variables that entered into the model (pollen: F = 8.32, *P* = 0.006; nectar: F = 3.32, *P* = 0.08, whole model: F = 2.82, *P* = 0.01). This multivariate analysis conducted for *B. laevigata* thus support the results found with univariate analyses in the same species, i.e. that nectar and pollen were the only variables that showed a significant correlation with visitation rates, and that this correlation is negative.

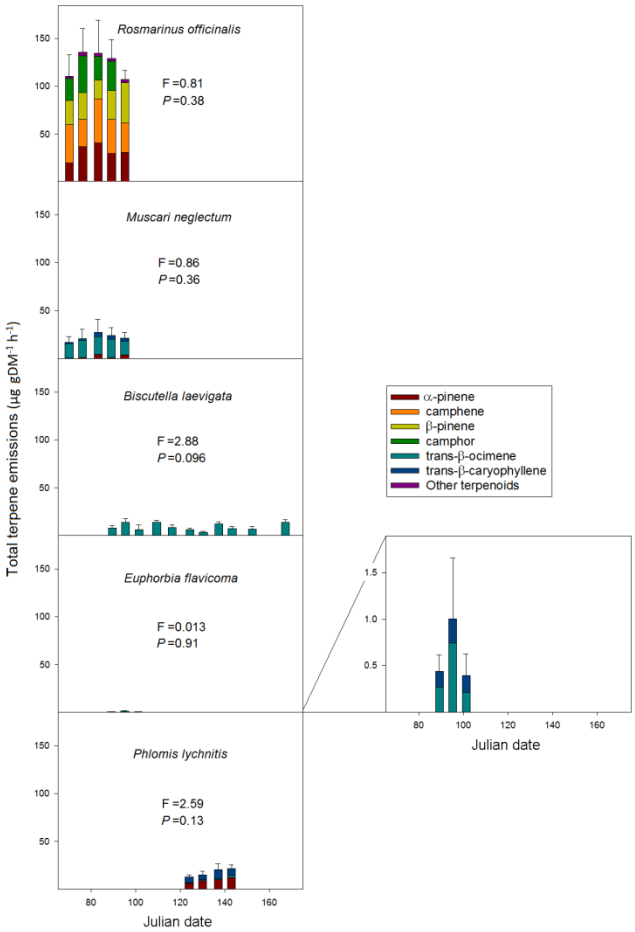


Figure 2. Mean values of the capacity of total and individual terpene emissions of the five plant species during their flowering periods in year 2011. Error bars indicate SEM (n = 5). F statistics and *P* values from the regression between number of flowers and day of the year are also depicted.

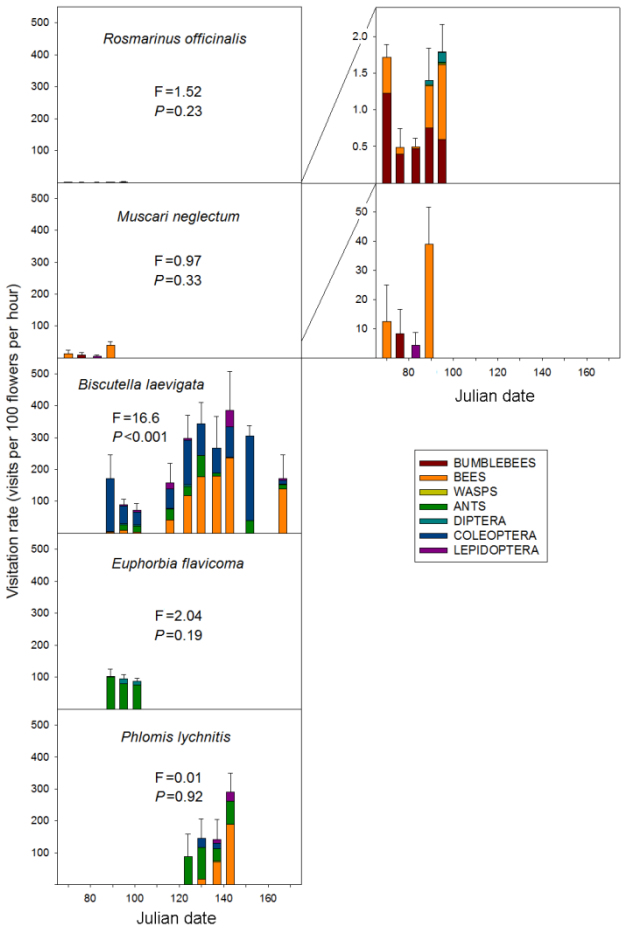


Figure 3. Mean values of insect visitation rates for the five plant species during their flowering periods in year 2011. Error bars indicate SEM (n = 5). F statistics and *P* values from the regression between number of flowers and day of the year are also depicted.

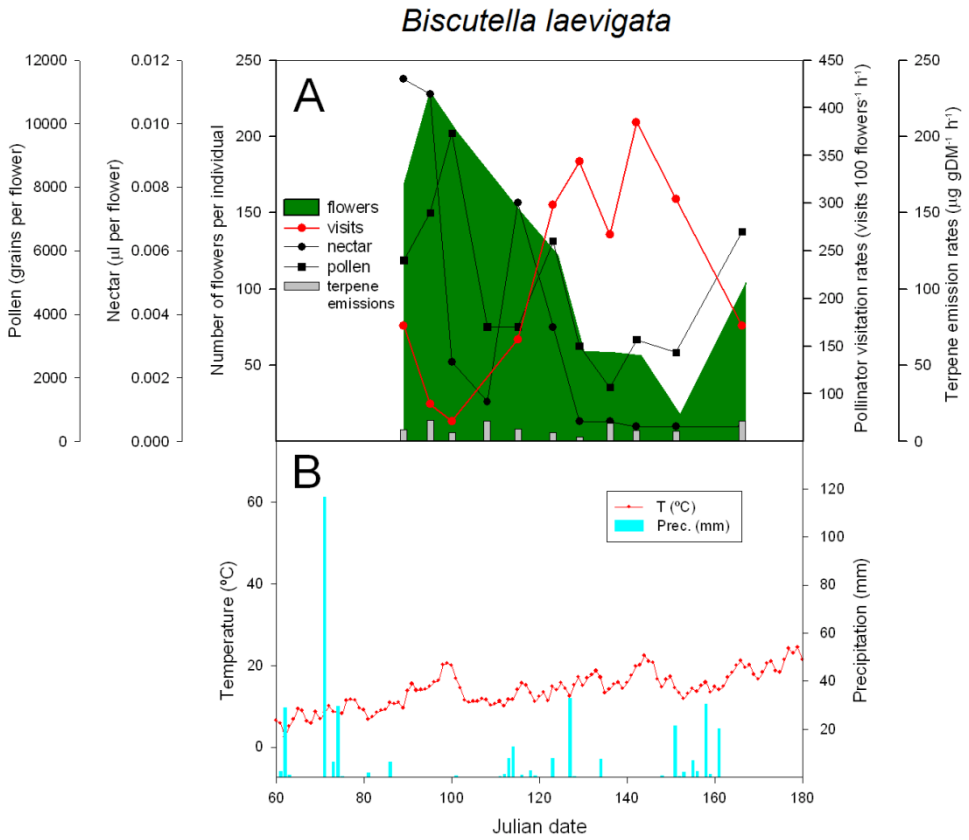


Figure 4. A, phenology of the number of flowers, nectar and pollen production, terpene emission rates, and pollinator visitation rates for *B. laevigata* in year 2011. Values are means of the five individuals sampled each week; B, mean temperature of the day (°C) and accumulated precipitation of the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

Discussion

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

We observed a similar temporal pattern between nectar and pollen production per flower in the long-period flowering species *B. laevigata* and nectar and pollen abundance at the community level as that measured by Filella *et al.* (2013) (Figure 4). These results support

our hypothesis that decreasing competitive pressure for the attention of pollinators drives a decrease in floral investment in rewards. The short-period flowering species, *R. officinalis*, *M. neglectum*, *E. flavicoma*, and *P. lychnitis*, did not develop patterns except for a trend to decrease nectar production in species that flowered in late spring and early summer. We observed a definite trend of decreasing production of rewards especially in the species with the longest flowering period, *B. laevigata*, which was the only species that completely included the main change in the plant-pollinator market within its flowering period.

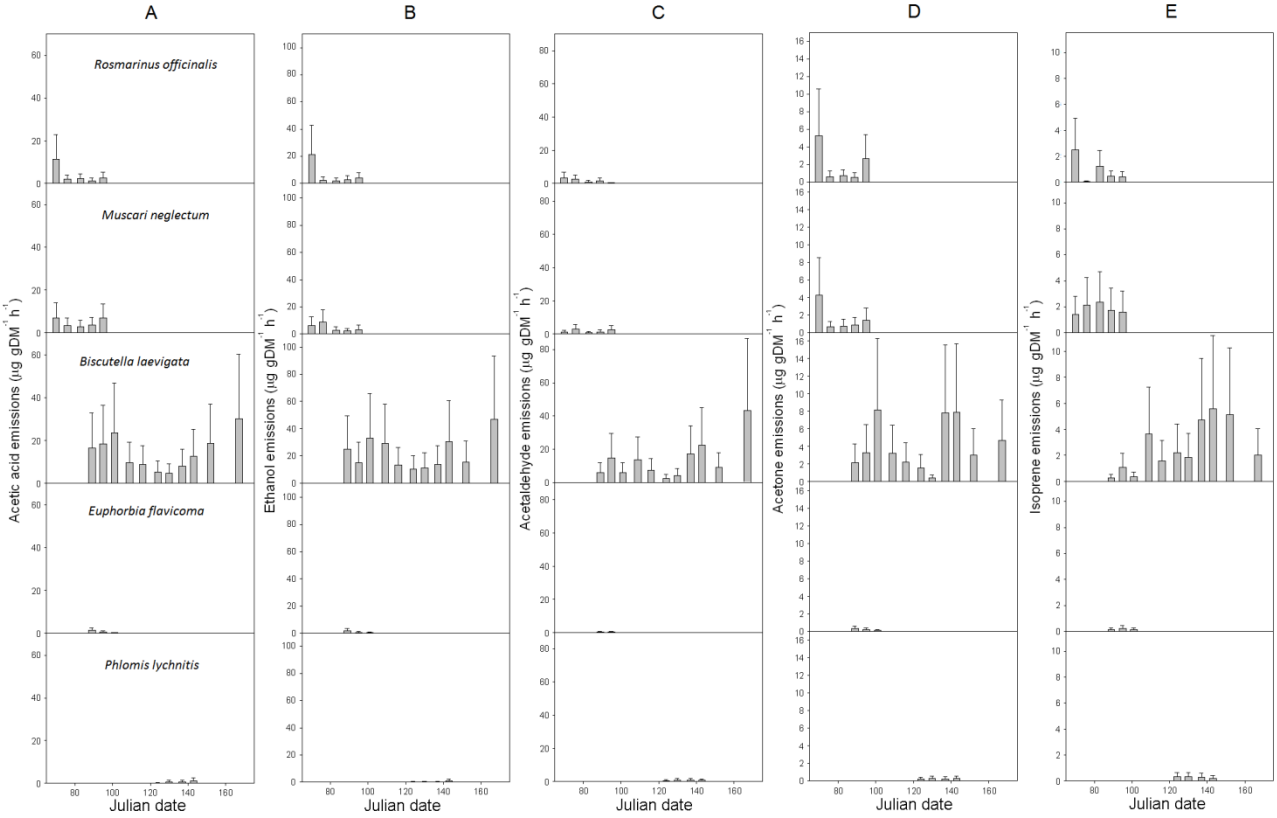
A significant negative correlation was found between floral rewards (nectar and pollen) and visitation rates in the case of *B. laevigata*. The pattern that we hypothesized for floral rewards was thus accomplished in this long-flowering species. These variables were differently affected by climatic conditions.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

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

In summary, while floral VOC emission capacities did not show a pattern of decrease in any species, the production of floral rewards generally decreased throughout the flowering period in *B. laevigata*, while the visitation rates of pollinators increased in this species. These results would support the hypothesis of a decreasing competitive pressure for the attention of pollinators that may drive a decrease in floral investment in insect rewards but not the hypothesis of a possible accompanying decrease of the capacity of emitting floral VOC olfactory signals. We detected these patterns in rewards and visits of pollinators especially in the species with the longest flowering period, *B. laevigata*, which experienced the main

changes in the plant-pollinator market. Nectar production decreased in *B. laevigata* and *E. flavicoma*. The visitation rates of pollinators increased in *B. laevigata* and *P. lychnitis*. Insect abundance and activity increased with temperature throughout spring and summer, but the capacity of plants to invest resources in nectar may also be negatively affected by drier conditions occurring in late spring and summer. The negative correlation between production of floral rewards and visitation rates may thus be also induced by climatic conditions making it difficult to discern possible evolutionary forces tending to decrease rewards when pollinator visitation rates are high.

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



Appendix 1. Mean values of: A, acetic acid emissions; B, ethanol emissions; C, acetaldehyde emissions; D, acetone emissions and E, isoprene emissions of the five plant species during their flowering periods obtained by dynamic headspace measurements conducted with PTR-MS. Error bars indicate SEM (n = 5).

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Chapter 4. Optimum temperature for floral terpene emissions tracks the mean temperature of the flowering season

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Abstract

Emissions of volatiles from leaves exhibit temperature dependence with maximums, but optimum temperatures for the release of floral volatiles or the mechanism of optimization have not been determined. We hypothesized that flowers have an optimum temperature for the emission of volatiles and, because the period of flowering varies highly among species, that this optimum is adapted to the temperatures prevailing during flowering. To test these hypotheses, we characterized the temperature responses of floral terpene emissions of diverse widespread Mediterranean plant species flowering in different seasons by using dynamic headspace sampling and analysis with gas chromatography mass spectrometry. The emissions from *Dittrichia viscosa* were sampled repeatedly during its flowering period to test the hypotheses at the species level. The floral emissions of terpenes across species exhibited maximums at the temperatures corresponding to the season of flowering, with the lowest optimal temperatures observed in winter-flowering and the highest in summer-flowering species. An analogous trend was evident for intraspecific emissions in the case of *Dittrichia viscosa*, with a lower optimum temperature for the measurements conducted in autumn than for those conducted in late summer. These trends were valid for emissions of both total terpenes and the various terpene compounds. The results show that the optimum temperature of floral volatile emissions scales with temperature at flowering and suggest that this scaling is the outcome of physiological adaptations of the biosynthetic and/or emission mechanisms of flowers.

Keywords: flower scent, interspecific variation, phenology, seasonal variability.

Introduction

Floral emissions of volatile organic compounds (VOCs) constitute important olfactive signals for pollinators to locate and identify flowers and thus mediate pollination in entomophilous angiosperms (Dudareva *et al.*, 2006). Floral emissions, however, are susceptible to diverse biotic and abiotic factors that can lead to significant changes in emission rates and composition, thereby interfering with or affecting chemical communication between plants and pollinators (Farré-Armengol *et al.*, 2013, 2014). Several environmental factors can affect the emission of VOCs from various plant tissues; the effects of temperature and light on foliar terpene emissions are the best studied (Peñuelas & Llusià, 2001; Niinemets *et al.*, 2004; Grote *et al.*, 2013). The responses of terpene emissions from leaves to temperature are well characterized (Niinemets *et al.*, 2010) and are known to be determined by temperature dependencies of the physicochemical properties of terpenes, such as volatility, solubility and diffusivity, and by the effects of temperature on foliar physiology, such as terpene biosynthesis or stomatal resistance (Reichstein *et al.*, 2002; Niinemets *et al.*, 2004; Harley, 2013). The responses of terpene emissions from flowers to temperature are less known. However, we argue here that the need of maximization of the intensity of floral olfactive signals to enhance the ability of pollinators to locate flowers has likely exerted a selective pressure on floral physiology to tune the maximum floral emissions to the temperature ranges to which the flowers of each species are typically exposed.

Species from cooler environments have lower optimum temperatures for photosynthesis than do species living in warmer environments, which reveals a positive correlation between species-specific optimum temperature for photosynthesis and the range of ambient temperatures in which the species live (Berry & Björkman, 1980; Niinemets *et al.*, 1999; Medlyn *et al.*, 2002). The optimum foliar temperature for photosynthesis also varies within species, depending on the range of temperatures under which individuals grow,

indicating an additional physiological process of acclimation (Cleveland *et al.*, 1992; Kattge & Knorr, 2007). In species that do not store terpenes, the rates of terpene emission have temperature response curves similar to those of photosynthesis (Copolovici & Niinemets, 2005; Llusia *et al.*, 2006; Niinemets *et al.*, 2010). In fact, terpene biosynthesis and physiological processes related to the emission of terpenes are affected by temperature in a way similar to that of photosynthetic rates. Moreover, the biosynthetic pathways responsible for the production of terpenes are dependent on the rates of carbon assimilation, and the acclimation of temperature responses of the rates of terpene emission has also been proposed (Staudt *et al.*, 2003; Niinemets, 2004). We hypothesized that plant species may thus be expected to experience adaptive trends to fine-tune the temperature responses of floral emissions to match the thermal environment the flowers typically encounter throughout the period of flowering. In this study, we aimed to test this hypothesis in Mediterranean species flowering at different times of the year.

Most Mediterranean angiosperms flower in spring. Some species, however, flower in summer, autumn or even winter. Flowers are thus exposed to different temperature ranges and can potentially evolve different temperature sensitivities of their floral emissions. The flowers of winter-flowering species are exposed to low temperatures and therefore are expected to adapt their optimal floral emissions to low temperature ranges. In contrast, summer-flowering species may adapt their floral emissions to high temperatures. Such different responses can result from differences in the composition of volatiles emitted by the species and from physiological modifications in the production and release of volatiles.

We tested the key hypothesis that optimum temperatures maximizing floral terpene emissions depend on the temperatures prevailing during the flowering period. The hypothesis was tested with seven Mediterranean species flowering at different times of the year. We also sampled terpene emissions at two different times during the flowering period in the Mediterranean perennial herb *Dittrichia viscosa* to explore whether the optimum temperatures for floral emissions can also vary within species having prolonged flowering periods extending over widely differing temperatures.

Materials and methods

Study site and species sampled

The study was conducted at various field locations within the province of Barcelona (Catalonia, Spain). Seven common Mediterranean species of plants in Garraf national park (*Dorycnium pentaphyllum* Scop., *Erica multiflora* L., *Globularia alypum* L.), Collserola national park (*Quercus ilex* L.) and Cerdanyola del Vallès (*Spartium junceum* L., *Sonchus tenerrimus* L., *Dittrichia viscosa* (L.) Greuter) were included in the analysis. Floral emissions from *D. viscosa* were collected in late summer and again in early autumn. The species sampled include a wide range of flowering periods with different mean temperatures (Table S1, Suppl. Mat.).

Temperature-response curves

Samples of emissions were collected using a dynamic headspace technique. A portable infrared gas analyzer (IRGA) system (LC-Pro+, ADC BioScientific Ltd., Great Amwell) was employed to measure gas exchange and to provide a constant light intensity of 1000 µmol m^{-2} s^{-1} and the required temperatures. The temperature responses of floral emissions were measured in the field over a range of temperatures of 15-40°C at intervals of 5°C. The IRGA

system used reached a maximum temperature of 40°C. The maximum temperature reached in the winter measurements, however, was only 30°C because the IRGA system was unable to heat the ambient air to higher temperatures.

One or several attached flowers were enclosed in the chamber of the IRGA (*G. alypum*: 1 capitula, *E. multiflora*: 8-12 flowers, *Q. ilex*: 1 male inflorescence, *D. pentaphyllum*: 10-15 flowers, *S. junceum*: 4-5 flowers, *S. tenerrimus*: 1 capitula, *D. viscosa*: 5-9 capitula). We used two different chambers depending on the size of the flowers of each species. A 12 cm^3 chamber was used for *G. alypum*, *E. multiflora*, *Q. Ilex*, *D pentaphyllum* and *S. tenerrimus*, and a 175 cm^3 chamber was used for *S. junceum* and *D. viscosa*. We collected the samples of terpene emissions after setting the required quantum flux density and temperature and after an acclimation period of approximately 10 min or the time needed to reach a steady-state exchange of CO_2 and H_2O. The enclosed flowers were sequentially submitted to different temperatures, and their emissions were sampled for additional 10 min. The air exiting the chamber of the IRGA, at a mean flux of air of approximately 200-250 ml min^{-1}, was directed through a Teflon tube to a tube filled with the adsorbents Tenax (114.6 mg , 50% vol.) and Carbotrap (236.8 mg, 50% vol.), which collected the terpenes emitted by the flower(s) over a period of 10-15 min. The same process was repeated with empty chambers of the IRGA that served as blanks of the system. At least two blank samples were collected for each curve, one at the beginning of the emission samplings and another at the end. We collected 3-5 replicate samples of emissions per species (*G. alypum*: 5, *E. multiflora*: 4, *Q. ilex*: 4, *D. pentaphyllum*: 5, *S. junceum*: 5, *S. tenerrimus*: 4, *D. viscosa* late summer: 3, *D. vicosa* early autumn: 3). Each replicate was collected from a different plant. At the end of each sampling sequence we collected the flower samples from which emissions were collected and we dried and weighed the flowers for emission rate calculations.

Terpene analyses

The terpene samples in the adsorbent tubes were thermally desorbed, and the samples were analyzed by an Agilent gas cromatography mass spectrometry (GC-MS) system (Agilent Technologies, GC: 7890A, MS: 5975C inert MSD with Triple-Axis Detector, Palo Alto, CA, USA). Samples were injected into a 30 m x 0.25 mm x 0.25 µm capillary column (HP-5MS, Agilent Technologies). Helium flow was 1 ml min^{-1}, and total run time was 26 min. After injection, the sample was maintained at 35°C for 1 min, the temperature was then increased at 15°C min^{-1} to 150°C and maintained for 5 min, then increased at 50°C min^{-1} to 250°C and maintained for 5 min and then increased at 30°C min^{-1} to 280°C and maintained for 5 min.

The terpenes were identified by comparing the retention times with standards from Fluka (Buchs, Switzerland) that had been injected into clean adsorbent tubes, and the fractionation mass spectra were compared with standard spectra and spectra in the Nist05a and wiley7n mass spectral libraries. Terpene concentrations were determined from the calibration curves. Calibration curves for the common terpenes α-pinene, β-pinene, limonene, γ-terpinene, linalool and α-humulene were determined daily. The terpene calibration curves (n=4 different terpene concentrations) were always highly significant (R^2>0.99 for the relationship between the signal and the amount of compound injected).

Statistical analysis

We used the *loess* function of the R software package (R Development Core Team, 2011) to characterize the shape of the curve of the temperature responses of floral terpene emissions.

The *loess* function fits local polynomial functions to the data in different ranges of the independent variable (Cleveland *et al.*, 1992). We used SigmaPlot 11.0 to visualize the data and to determine the relationship between optimum temperature for floral emissions and mean temperature of the month of the flowering peak by linear regression models.

Optimum temperature for floral emissions

The mean ambient temperature for the month of the flowering peak for each species in the region from which the species was sampled was calculated as the average for the period 1971-2000 (Servei Meteorològic de Catalunya, 2010). The optimum temperatures for floral emissions of each species were obtained from the maxima of the fitted temperature-response curves. Optimum temperatures for each terpene present in the floral emissions from each species were estimated as the temperatures at the highest emission of that compound.

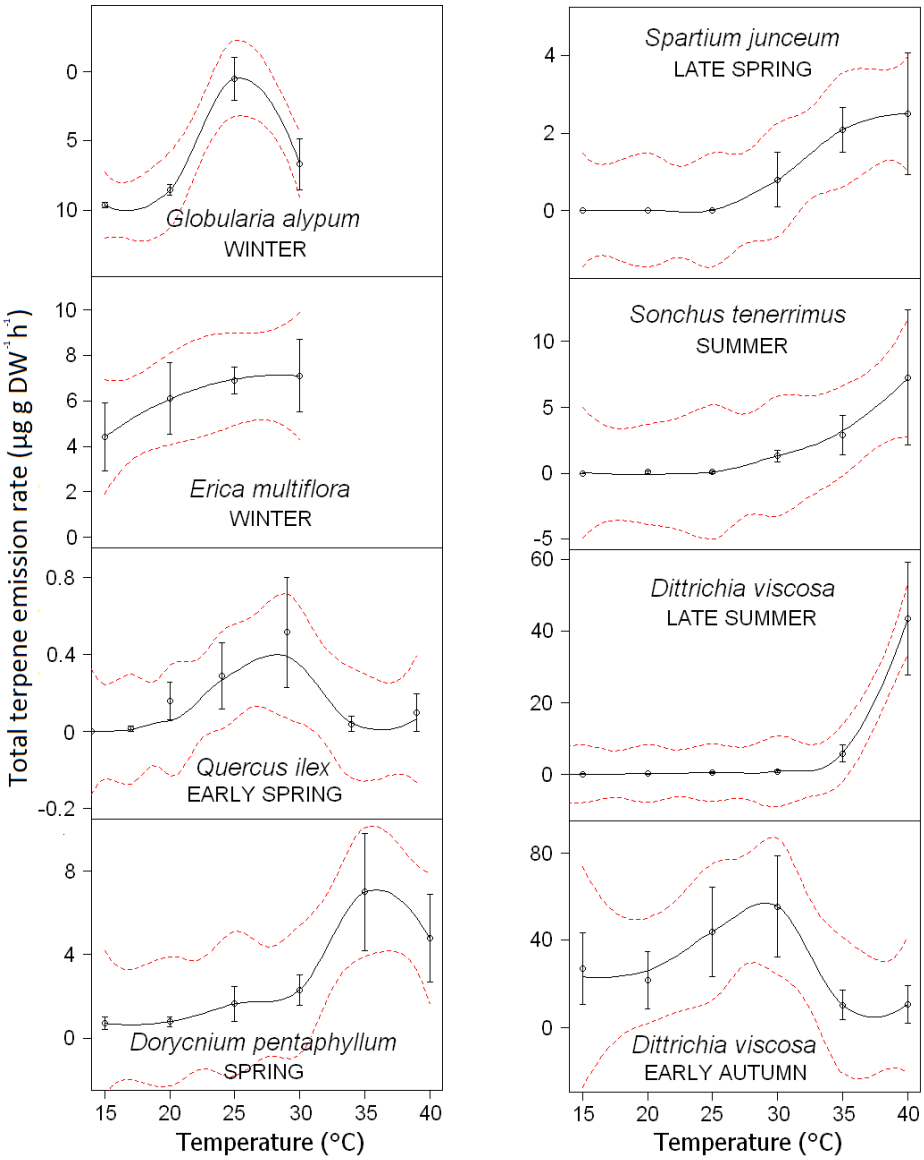

Figure 1. Rates of total terpene emission per dry weight of floral tissue (µg g DW^{-1} h^{-1}) throughout the temperature gradient from 15 to 40°C. The quantum flux density was maintained at 1000 µmol m^{-2} s^{-1} during the measurements. The data were fitted by local polynomial functions (discontinuous lines indicate the 95% confidence intervals). Error bars indicate SE (*n*=3-6 plants).

Results

Globularia alypum and *E. multiflora* flowers emitted detectable amounts of 1R-α-pinene, camphene, 3-carene and D-limonene (Table S2). *Quercus ilex* male flowers emitted 1R-α-pinene, β-pinene, camphene, 3-carene and D-limonene. *Dorycnium pentaphyllum* flowers emitted 3-carene, (E)-β-ocimene and (Z)-β-ocimene. *Spartium junceum* flowers emitted 1R-α-pinene and α-farnesene. *Sonchus tenerrimus* flowers emitted 1R-α-pinene and 3-carene. *Dittrichia viscosa* flowers of late summer emitted 1R-α-pinene, 1S-α-pinene, β-pinene, α-phellandrene, β-phellandrene, camphene, 3-carene, D-limonene, eucalyptol, γ-terpinene, α-terpinolene and α-thujene. *Dittrichia viscosa* flowers of early autumn emitted 1R-α-pinene, β-pinene, α-phellandrene, camphene, 3-carene and D-limonene (Table S2).

The rates of terpene emission initially increased with temperature in all species and generally reached a maximum (Figure 1). The temperature-response curves of floral terpene emissions showed species-specific differences. The rates of floral emission of winter-, autumn- and spring-flowering species began to decline at different temperatures, usually between 30 and 40°C, and the emissions from summer-flowering species did not decline within the range of temperatures included in our measurements. The winter-flowering species *G. alypum* and *E. multiflora* exhibited maximum floral terpene emissions at 25°C and 30°C, respectively. Floral emissions from *Q. ilex* reached a maximum at approximately 30°C. In the spring-flowering *D. pentaphyllum*, the rates of floral terpene emission increased with increasing temperature up to 35°C, and a moderate reduction was observed at 40°C. The rates of terpene emission in the flowers of *S. junceum*, *D. viscosa* and *S. tenerrimus* sampled in late spring and summer increased with increasing temperature, even up to 40°C, whereas the summer flowers of *D. viscosa* and *S. tenerrimus* experienced a maximum increase only from 35 to 40°C. In early autumn, the maximum emission from *D. viscosa* flowers was at 25-30°C (Figure 1).

The optimum temperature for floral emissions of all terpenes for each species were positively and linearly correlated with the mean temperature of the month of the flowering peak (P=0.002, Figure 2). Across the species sampled, the optimum temperatures for floral emissions of each terpene compound were also positively and linearly correlated with the mean temperature of the month of the flowering peak (α-pinene, P=0.02; camphene, P=0.03; β-pinene, P=0.17; 3-carene, P=0.008; D-limonene, P<0.001; FIgure 3).

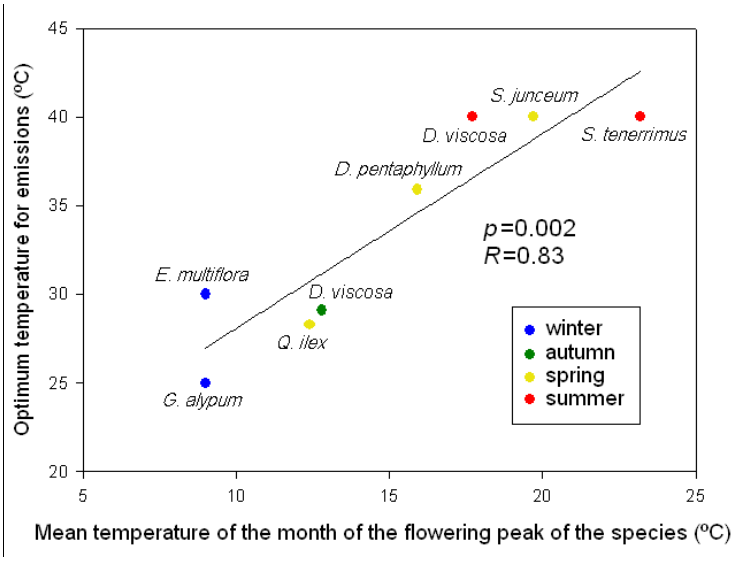

Figure 2. Relationships between the optimum temperature for floral emissions of terpenes and the mean temperature for the month of the flowering peak of the species. Colors indicate the flowering season of the species (blue, winter; green, autumn; yellow, spring; red, summer).

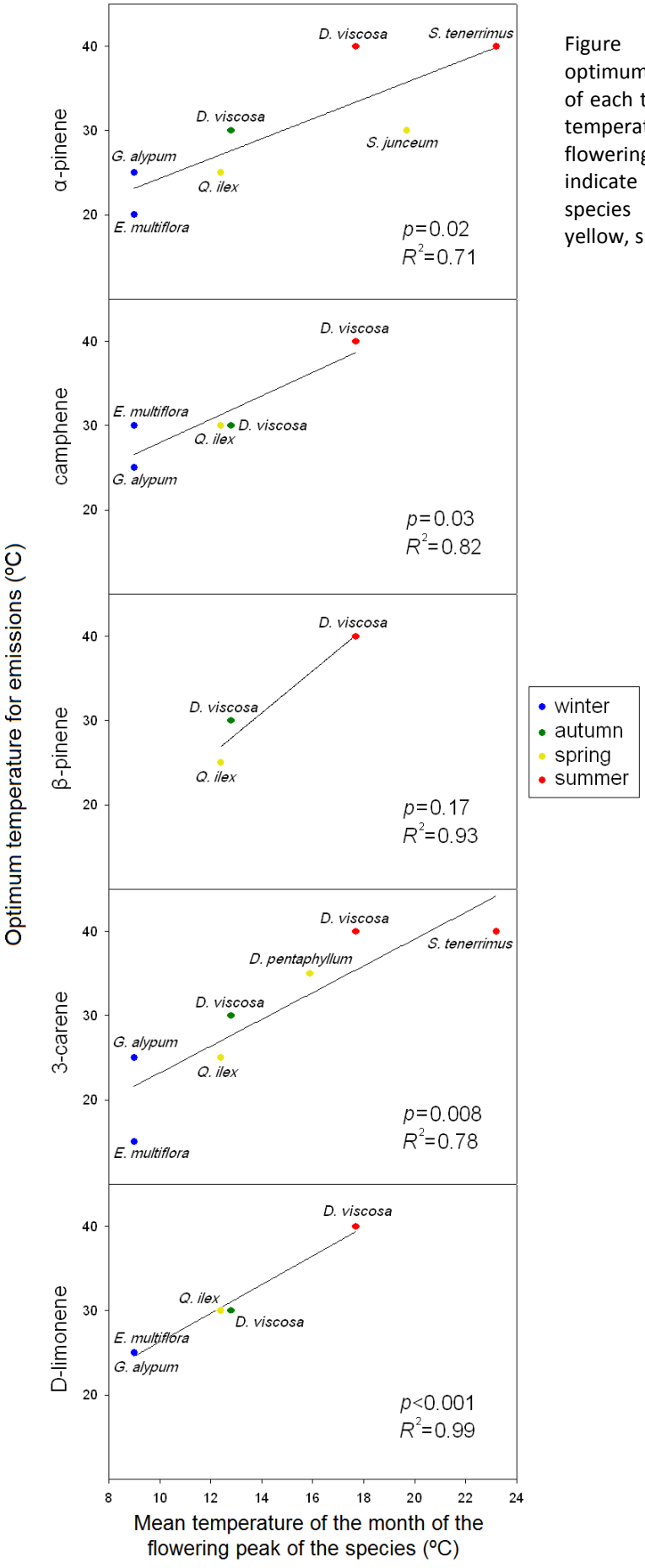

Figure 3. Correlations between the optimum temperature for floral emissions of each terpene compound and the mean temperature for the month of the flowering peak of the species. Colors indicate the flowering season of the species (blue, winter; green, autumn; yellow, spring; red, summer).

Discussion

Our data demonstrate that the well-known temperature-dependent increase of terpene emissions previously reported for leaves also occurs in flowers (Figure 1). The temperature responses of floral volatile emission generally exhibited an optimum, suggesting that these emissions reflect de novo synthesis of terpenes (Niinemets *et al.*, 2010; Li & Sharkey, 2013; Monson, 2013). The temperature dependence function for de-novo synthesized isoprenoids considers an Arrhenius type response which describes a curve with an optimum (Niinemets *et al.*, 2010). This optimum represents a threshold temperature from which physiological processes involved in isoprenoid biosynthesis are limited or completely inhibited. On the other hand, the emission rates for species that store monoterpenes in specialized plant tissues are suggested to be controlled only by physical evaporation and diffusion, two processes that do not decline but present a sustained increase with temperature.

As we hypothesized, species flowering in different seasons had optimum temperatures for floral emissions that paralleled the mean temperature of the month of the flowering peak (Figure 2). The positive correlation between the temperature optimum for floral emission and ambient temperature generally resembled the correlation between optimum temperature for photosynthesis and ambient temperature (Berry & Björkman, 1980; Niinemets *et al.*, 1999; Kattge & Knorr, 2007). Species flowering in cold seasons had maximum emissions at lower temperatures than did species flowering in warm seasons. Our results thus supported the hypothesis that the temperature responses of floral terpene emissions were adapted to the temperature ranges to which the flowers were exposed during flowering. Even though we were not able to determine the precise optimum temperature for floral emissions in summer species, we clearly demonstrated that it was above 40°C. If we could obtain the real optimum for these species, the difference between optimums for species flowering in cold and warm seasons would increase, strengthening the significance of our conclusions.

Our results also showed that the emission rates of each terpene compound also tended to have an emission optimum, and that this optimum was positively correlated with the mean temperature of the month of the flowering peak of that species (Figure 3). This response of the individual terpene compounds indicated that the differences in the optimum temperature for total terpene emissions among species was not due to the differences in the compounds that constitute the scents of flowers, but reflected physiological adaptation of underlying biochemical processes. Terpene production in summer-flowering species has thus been adapted such that floral terpene emissions are maximized at high temperatures and are strongly curbed at low temperatures. In contrast, terpene production in winter-flowering species has been adapted to maximize floral emissions at low temperatures. This pattern is clearly supported in the insect-pollinated species explored in this study. We only studied one wind-pollinated species, *Q. ilex*. *Quercus ilex* also fits into this pattern, indicating that adaptation of optimum temperature for floral terpene emissions to ambient temperature of the flowering season might not be exclusively linked to biotic pollination.

We observed different temperature responses of floral terpene emissions in *D. viscosa* in late summer and early autumn. Analogous intraspecific seasonal differences in the responses of terpene emissions to environmental conditions have been observed for leaves (Llusia *et al.*, 2006; Helmig *et al.*, 2013). These results suggest that temperature dependencies of floral emissions can vary even within individuals of the same species, at least in those species that can flower under different temperature conditions, and indicate some degree of phenotypic, epigenetic or genotypic plasticity in the physiology of the flowers of these species, which clearly constitutes an important adaptive modification to optimize flower emissions at diverse temperature ranges.

Such plasticity in the physiology of flowers controlling terpene floral emissions could be adaptations of the terpene biosynthetic and/or release mechanisms of floral volatiles. The biosynthetic pathways involved in volatile terpene production are well described (Dewick, 2002; Dubey *et al.*, 2003; Kuzuyama & Seto, 2003), and the mechanisms that regulate terpene biosynthetic rates have been extensively investigated (Dudareva & Pichersky, 2000; Fischbach *et al.*, 2002; Dudareva *et al.*, 2004; van Schie *et al.*, 2006). The key controls operating in terpene production are the transcription, production and activity of enzymes and the concentrations of the substrates of these enzymes (Dudareva & Pichersky, 2000; Fischbach *et al.*, 2002; Dudareva *et al.*, 2004; van Schie *et al.*, 2006). On the other hand, some mechanisms that mediate and control terpene release (e.g. stomatal closure, compound volatility and mechanisms of transport of terpenes across the cell) can regulate the rates of diffusion from internal terpene pools to the exterior and can thereby also limit the rates of terpene release by direct regulation of the resistance to terpene diffusion from the sites of synthesis to the external gas phase (Dudareva *et al.*, 2004). The convergent modifications in temperature adaptation of floral terpene release demonstrate a very high temperature-driven plasticity of plant physiological traits and clearly emphasize the need to consider genotypic, epigenetic and phenotypic plasticity in estimating and modeling floral emissions.

Conclusions

Our data demonstrate important variation in the temperature dependencies of floral terpene emissions. In particular, the lower optimum temperatures for emission maximum observed in species flowering in colder seasons and the higher optimum temperatures observed in species flowering in warmer seasons indicate species-specific temperature responses. This relationship suggests an adaptive mechanism that tunes floral emissions to match the temperatures to which the species are exposed during their flowering season. Furthermore, our results also show this adaptive trend among individuals of the same species, for example in *D. viscosa*, a species that has a long flowering period and that was sampled in late summer and early autumn. This observed seasonal change in the physiology of floral scent emission within a species indicates intraspecific plasticity and can constitute an additional major source of variability in floral emissions in the field. New measurements are warranted at different points in time in species with long flowering periods or with separate flowering periods throughout the year to gain a more detailed insight into the intraspecific plasticity of the physiology of flowers under different temperatures.

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Chapter 5. Removal of floral microbiota reduces floral terpene emissions

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Abstract

The emission of floral terpenes plays a key role in pollination in many plant species. We hypothesized that the floral phyllospheric microbiota could significantly influence these floral terpene emissions because microorganisms also produce and emit terpenes. We tested this hypothesis by analyzing the effect of removing the microbiota from flowers. We fumigated *Sambucus nigra* L. plants, including their flowers, with a combination of three broad-spectrum antibiotics and measured the floral emissions and tissular concentrations in both antibiotic-fumigated and non-fumigated plants. Floral terpene emissions decreased by ca. two thirds after fumigation. The concentration of terpenes in floral tissues did not decrease, and floral respiration rates did not change, indicating an absence of damage to the floral tissues. The suppression of the phyllospheric microbial communities also changed the composition and proportion of terpenes in the volatile blend. One week after fumigation, the flowers were not emitting β-ocimene, linalool, epoxylinalool, and linalool oxide. These results show a key role of the floral phyllospheric microbiota in the quantity and quality of floral terpene emissions and therefore a possible key role in pollination.

Introduction

Proficient performance in plants is strongly associated with distinct microbial communities that live in and on the organs. These communities are especially important in roots (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012). The microbiotas of the phyllosphere (in above-ground plant tissues and on above-ground plant surfaces) are abundant and are assumed to play critical roles in protecting plants from diseases and in promoting growth by various mechanisms. They may also offer indirect protection against pathogens (Arnold *et al.*, 2003; Vorholt, 2012; Bulgarelli *et al.*, 2013) and contribute to plant communication with different types and quantities of biogenic volatile organic compounds (BVOCs) (Vorholt, 2012; Bulgarelli *et al.*, 2013). Microbiotas, however, have generally not been well characterized, and little is known about their actual physiological and ecological roles (Lindow & Brandl, 2003; Vorholt, 2012; Bulgarelli *et al.*, 2013; Rastogi *et al.*, 2013; Peñuelas & Terradas, 2014). The composition and physiological and ecological roles are much less well known for the microbiotas in and on flowers than for those in and on leaves. Microorganisms produce and emit many BVOCs including several terpenes (Peñuelas *et al.*, 2014), so we hypothesized that floral phyllospheric microbiotas could significantly contribute to the emission of BVOCs, including terpenes, that play a key role in attracting pollinators (Farré-Armengol *et al.*, 2013). Here we tested this hypothesis by studying the floral emissions of *Sambucus nigra* L. flowers before and after removal of their floral microbiota with a combination of three broad-spectrum antibiotics: streptomycin, oxytetracycline, and chloramphenicol.

Materials and Methods

Plant material and experimental setup

We used twenty flowering four-year-old potted *S. nigra* plants grown in a nursery (Tres Turons S.C.P., Castellar del Vallès, Catalonia, Spain) outdoors under ambient Mediterranean conditions. They were grown in 15-L pots with a substrate of peat and sand (2:1) and received regular irrigation, ensuring that the substrate was held at field capacity throughout the experiment. Ten plants were fumigated with antibiotics. The plants were fumigated with 1600 ppm streptomycin, 400 ppm oxytetracycline, and 200 ppm chloramphenicol in 50 ml of H_2O

with 1% glycerol to ensure the elimination of floral phyllospheric microbiota. These antibiotics are used in agriculture mainly in prophylactic treatments (Vidaver, 2002). The other ten plants served as controls and were fumigated with 50 ml H_2O with 1% glycerol but without antibiotics. The terpenes in both floral emissions and contents of the control and fumigated plants were measured at day −2 (pre-treatment, two days before fumigation) and at days 2 and 8 (post-treatment) with a dynamic headspace technique.

Measurements of CO_2 and BVOC exchange

Floral CO_2 and H_2O exchanges were measured with the LCpro+ Photosynthesis System (ADC BioScientific Ltd., Herts, England) at standard conditions of temperature (30˚C) and light (PAR=1000 µmol m^{-2} s^{-1}). Several flowers from one inflorescence were enclosed in the chamber (175 cm^3) without detaching the flowers from the plant. In order to determine and quantify BVOC exchange, flow meters were used to monitor the air entering and exiting the floral chamber and system blanks were sampled previous and after each sampling. The air exiting the chamber was then analyzed by proton transfer reaction mass spectrometry (PTR-MS; Ionicon Analytik, Innsbruck, Austria) to calculate monoterpene emission rates. Every 15 minutes, the output air flowing from the leaf chamber was also sampled for 10 additional minutes using stainless steel tubes filled with VOC adsorbents. Thereafter, the adsorbed terpenes were analyzed by thermal desorption and gas chromatography-mass spectrometry (GC-MS) to characterize the relative concentration of each single terpene. The floral terpene emissions were calculated from the difference between the concentration of terpenes from chambers clamped to flowers and the concentration from chambers with no flowers and adjusted with the flow rates. A Teflon tube connected the chamber to the PTR-MS system (50 cm long and 2 mm internal diameter). The system used was identical for all measurements. The flowers measured in each sample-replicate were collected each sampling day, after finishing the measurements, and dried into an oven at 70˚C until constant weight to get the dry weight of the floral emitting sample.

PTR-MS

PTR-MS is based on chemical ionization, specifically non-dissociative proton transfer from H_3O^+ ions to most of the common BVOCs, and has been fully described elsewhere (Lindinger *et al.*, 1998; Peñuelas *et al.*, 2005). In our experiment, the PTR-MS drift tube was operated at 2.1 mbar and 50˚C, with an E/N (electric field/molecule number density) of approximately 130 Td (townsend) (1 Td = 10^{-17} V cm^2). The primary ion signal (H_3O^+) was maintained at approximately 6×10^6 counts per second. The instrument was calibrated using an aromatic mixed-gas standard (TO-14A, Restek, Bellefonte, PA, USA) and a monoterpene gas standard (Abello Linde SA, Barcelona). Masses 155, 137 and 81 were continuously monitored to calculate monoterpene emission rates.

Terpene sampling and analysis by GC-MS

Exhaust air from the chambers was pumped through a stainless steel tube (8 cm long and 0.3 cm internal diameter) (Markes International Inc. Wilmington, USA) filled manually with the VOC adsorbents (115 mg of Tenax® and 230 mg of Unicarb®, Markes International Inc. Wilmington, USA) separated by a metallic grid. Samples were collected using a QMAX air-sampling pump (Supelco Inc., Bellefonte, PE, USA). For more details, see Peñuelas *et al.* (2005, 2013). The sampling time was 10 min, and the flow varied between 100 and 200 mL min^{-1},

depending on the adsorbent. The tubes were stored at 228˚C until the analysis. We also prepared extracts of each floral sample for the posterior analysis of the floral volatile concentrations with GC-MS. We froze the samples in liquid nitrogen and ground them in vials with 500 µL of pentane that served as a solvent for the extracted contents.

Terpene analyses were performed using a gas chromatograph (7890A, Agilent Technologies, Santa Clara, USA) with a mass spectrometric detector (5975C inert MSD with Triple-Axis Detector, Agilent Technologies). The terpenes trapped in the tubes were processed with an automatic sample processor (TD Autosampler, Series 2 Ultra, Markes International Inc. Wilmington, USA) and desorbed using an injector (Unity, Series 2, Markes International Inc. Wilmington, USA) into a 30 m^3 0.25 mm^3 0.25 µm film capillary column (HP-5ms, Agilent Technologies INC). The chromatographic program used for the identification and quantification of the terpenes is described in detail in Peñuelas *et al.* (2013). For pre-desorption and desorption, the flow was 50 ml min^{-1}, the split 10 ml min^{-1}, and the desorption temperature 330˚C.

Data analysis

The changes in the composition of the floral terpene emissions and concentrations were analysed by PERMANOVAs with Euclidean distances. The PERMANOVA analyses were conducted with R software (R Core Team, 2013) using the *adonis* function of the *vegan* package (Oksanen *et al.*, 2013). Statistica v8.0 (StatSoft) was used to perform the ANOVAs. Percentages were transformed to the arcsine of the square root previous to the ANOVA analyses comparing control and antibiotic-fumigated flowers.

Results

Reduced diversity and rates of emission of floral terpenes

The total floral emissions of terpenes decreased after antibiotic fumigation by nearly two thirds (Figure 1a). The flowers of *S. nigra* emitted a terpene mixture dominated by linalool, with lower emission rates of (Z)-β-ocimene and two oxygenated terpenes derived from linalool, epoxylinalool and linalool oxide (Figure 2a). The composition of the emissions significantly changed after fumigation (pseudo-F = 6.66, P = 0.05) (Figure 2a). The percentage of trans-β-ocimene decreased from 7 to 0.4% (F = 10.05, P < 0.05) by day 2. By day 8, trans-β-ocimene, linalool, epoxylinalool, and linalool oxide were not emitted by the fumigated flowers (Figure 2a).

Unaltered floral terpene contents

In contrast with terpene emissions, the terpene concentrations of floral extracts did not change in the fumigated plants (Figure 1b). Floral respiration rates were also not altered by fumigation (Figure 1c), indicating an absence of plant damage. Even though the emission rates of floral terpenes were high, ranging between 50 and 250 mg gDW^{-1} h^{-1} (Figure 1a), the terpene concentrations of floral extracts ranged from 0.5 to 2.5 mg gDW^{-1} (Figure 1b), indicating an absence of storage. The extracts were mainly dominated by epoxylinalool (Figure 2b). Linalool and linalool oxide were also present, although in low amounts, two days before and after fumigation but were not detected on day 8 (Figure 2b). The compositions of the

terpene contents were not significantly different between fumigated and control flowers (Figure 2b).

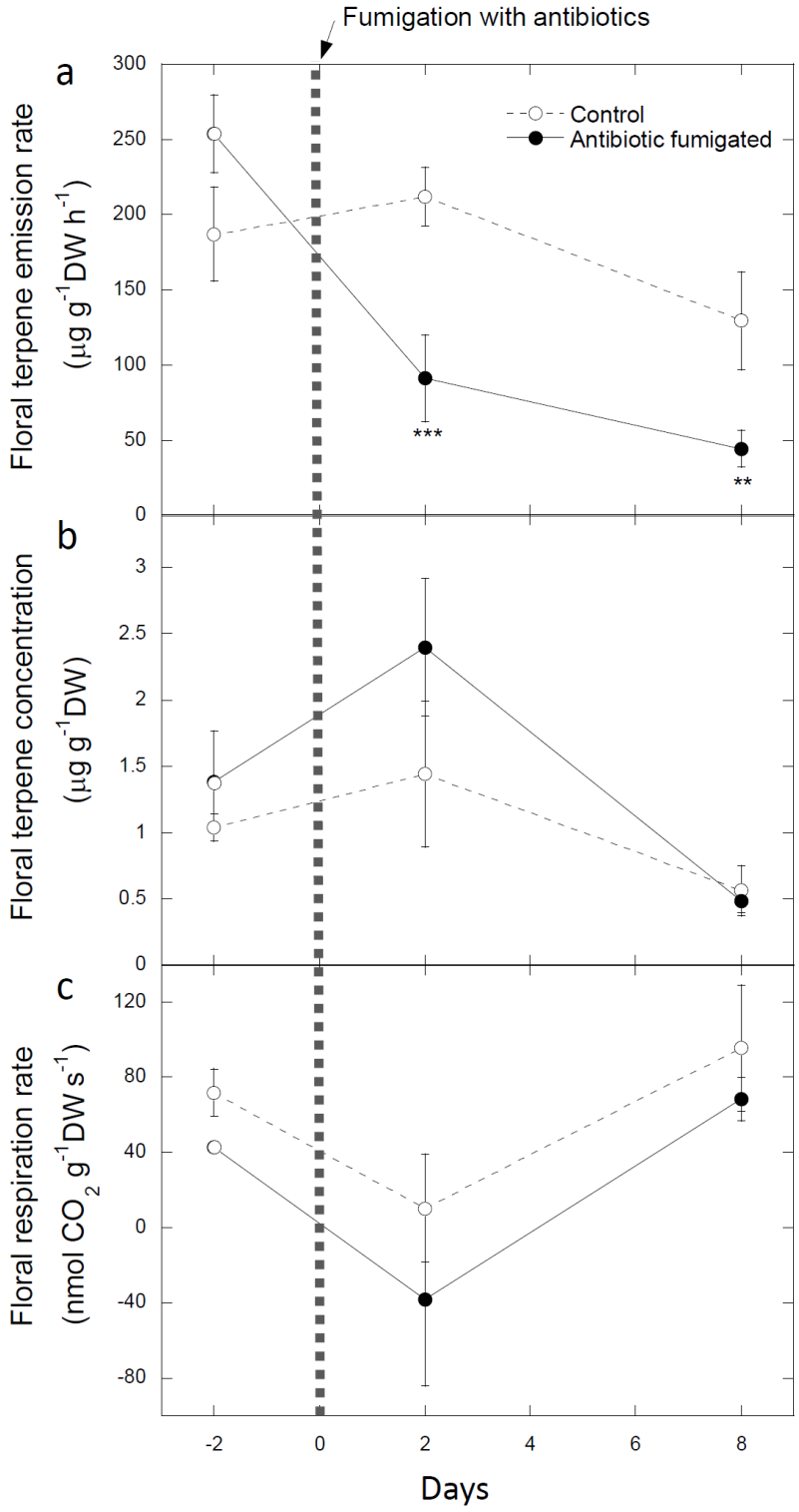

Figure 1. Effects of antibiotic fumigation on floral total terpene emissions, total terpene concentrations in floral tissues, and respiration. Time course of floral terpene emission rates (a), floral terpene concentrations (b), and respiration rates (c) of control and antibiotic fumigated *Sambucus nigra* plants. The antibiotics were applied to treated plants on day 0. The error bars are 6 SE (n 5 9). ** and *** indicate significant differences between control and antibiotic-fumigated flowers (ANOVA) at P , 0.01 and P , 0.001, respectively.

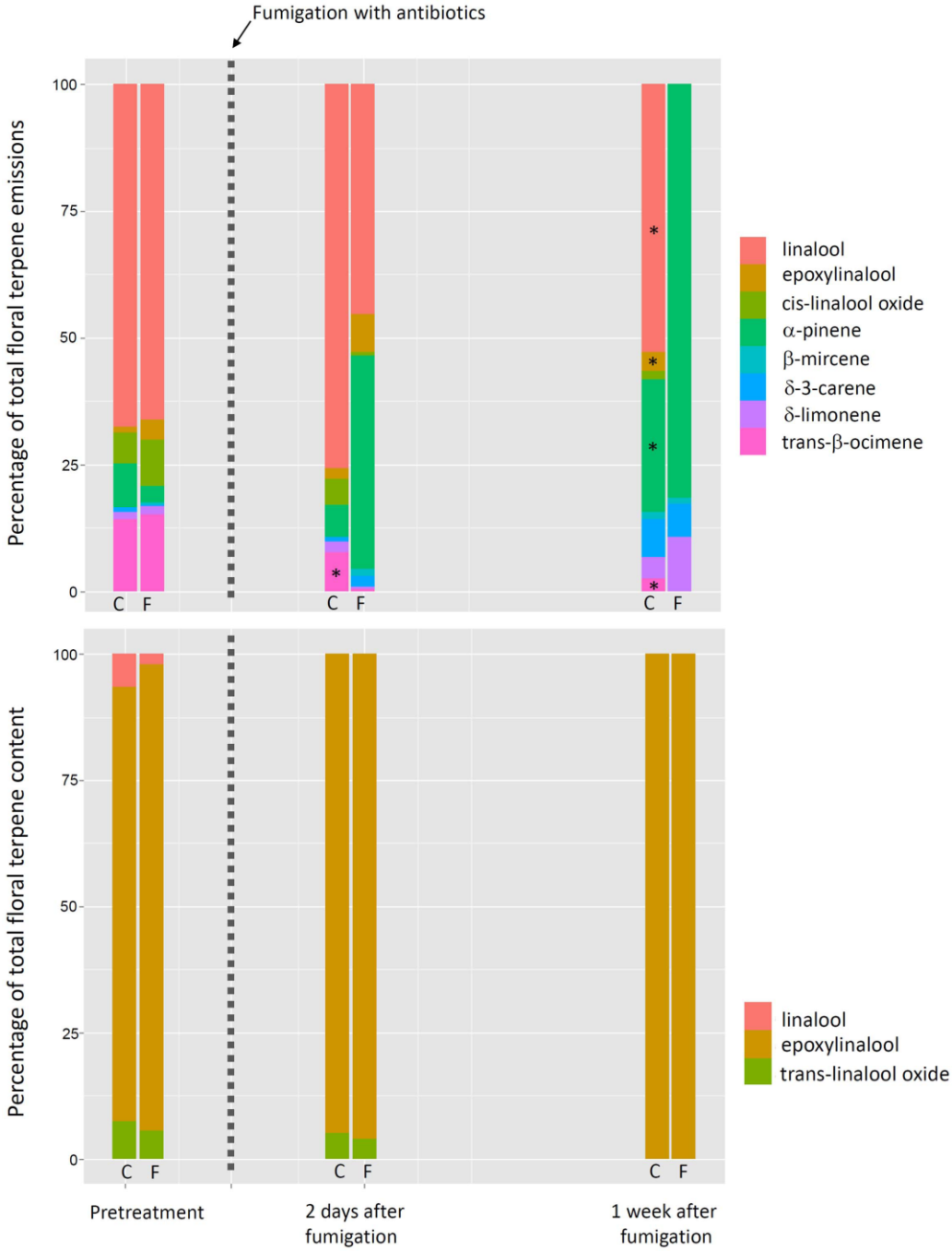

Figure 2. Effects of antibiotic fumigation on the composition and contents of floral terpene emissions. Time course of terpene composition of floral terpene scents (a) and floral terpene contents (b) of control and antibiotic-fumigated Sambucus nigra plants. The antibiotics were applied to treated plants on day 0. * indicates a significant difference (P , 0.05) between control and antibiotic-fumigated flowers (ANOVA).

Discussion

The reductions in the rate and diversity of floral terpene emissions in antibiotic-fumigated flowers were not due to a decrease in floral terpene contents. The functioning of the floral tissues did not appear to be altered, as indicated by the unaltered floral respiration rates. The decrease in emissions was thus likely due to the effect of the antibiotics on the floral

phyllospheric microbiota. Bacteria and fungi emit volatile organic compounds from de novo biosynthesis (Schulz & Dickschat, 2007; Davis *et al.*, 2013; Lemfack *et al.*, 2014) and biotransformation (de Carvalho & da Fonseca, 2006; Mirata *et al.*, 2008; Ponzoni *et al.*, 2008), including linalool and other terpenes (Raguso & Pichersky, 1999; de Carvalho & da Fonseca, 2006; Schulz & Dickschat, 2007; Peñuelas *et al.*, 2014). Terpene biosynthesis is well known in microbial metabolism, even though only a few bacterial and fungal genes encoding terpene synthases have yet been reported, likely due to the low aminoacid-sequence identities with homologous enzymes in eukaryotes (Peñuelas *et al.*, 2014). β-ocimene and linalool are emitted by yeasts from the genera *Debaryomyces*, *Kluyveromyces*, and *Pichia* (Ponzoni *et al.*, 2008), which are commonly found in the nectar of flowers (Sandhu & Waraich, 1985).

The emitted bouquet of *S. nigra* was dominated by linalool (3,7-dimethyl-1,6-octadien-3-ol), an acyclic monoterpene with a sweet, pleasant fragrance common in floral scents (Knudsen *et al.*, 1993). The dominant volatile in the floral extract, however, was epoxylinalool. De-epoxidation to linalool is favored at moderately low pH (Pfundel *et al.*, 1994), so the frequent presence of phyllospheric microorganisms producing extracellular acidic compounds (Müller & Seyfarth, 1997; Zwielehner *et al.*, 2008), along with the likely action of microbial epoxide hydrolases (Steinreiber & Faber, 2001), may have favored the emission of linalool.

Other possible effects of the antibiotic treatment, however, cannot be discarded. For example, the presence of certain phyllospheric microbes can induce an emission of defensive terpenes from flowers to control microbial communities (Arnold *et al.*, 2003). We could thus hypothesize that the removal of phyllospheric microbiotas could have temporarily released the plants from the need to maintain this defensive response, thus reducing the emissions. Direct interference of antibiotics with plant terpene synthesis, their reactions with terpenes, or the release of hydroxyl radicals from dying bacteria by bactericidal antibiotics cannot be fully discarded either.

Flowering plants use diverse, multifunctional biosynthetic pathways to produce a broad spectrum of BVOCs that collectively confer characteristic fragrances to flowers (Dudareva *et al.*, 2000). The results of this study highlight the mostly neglected role of phyllospheric microbiota in these emissions. The attractiveness of floral emissions to a wide range of pollinators, herbivores, and parasitoids and thus the key role emissions play in reproduction and defense may ultimately be due to the direct or indirect action of floral phyllospheric microbiota.

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Chapter 6. Enhanced emissions of floral volatiles by *Diplotaxis erucoides* (L.) in response to folivory and florivory by *Pieris brassicae* (L.)

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Abstract

The main function of floral emissions of volatile organic compounds (VOCs) in entomophilous plants is to attract pollinators. Floral blends, however, can also contain volatile compounds with defensive functions. These defensive volatiles are specifically emitted when plants are attacked by pathogens or herbivores. We characterized the changes in the floral emissions of *Diplotaxis erucoides* induced by folivory and florivory by *Pieris brassicae*. Plants were continually subjected to *folivory*, *florivory* and *folivory+florivory* treatments for two days. We measured floral emissions with proton transfer reaction/mass spectroscopy (PTR-MS) at different times during the application of the treatments. The emissions of methanol, ethyl acetate and another compound, likely 3-butenenitrile, increased significantly in response to florivory. Methanol and 3-butenenitrile increased 2.4- and 26-fold, respectively, in response to the *florivory* treatment. Methanol, 3-butenenitrile and ethyl acetate increased 3-, 100- and 9-fold, respectively, in response to the *folivory+florivory* treatment. Folivory alone had no detectable effect on floral emissions. All VOC emissions began immediately after attack, with no evidence of delayed induction in any of the treatments. Folivory and florivory had a synergistic effect when applied together, which strengthened the defensive response when the attack was extended to the entire plant.

Key Words: Methanol, glucosinolates, ethyl acetate, floral scent, VOCs, folivory-florivory synergy.

Introduction

Flowers are visited by many organisms that can produce positive, neutral or negative effects (Irwin *et al.*, 2004). Such visits can have important repercussions on plant fitness (Soper Gorden, 2013). The main visitors to flowers can be classified as pollinators, larcenists (nectar thieves) and florivores. Pollinators have positive effects on flowers by acting as effective vectors of pollination (Dafni, 1992; Dafni *et al.*, 2005), but larcenists and florivores have detrimental effects on flowers (Mothershead & Marquis, 2000; Field, 2001; Irwin *et al.*, 2001). Larcenists affect plant fitness negatively by exploiting and exhausting floral rewards, which are produced to attract pollinators, without contributing to successful pollination (Irwin *et al.*, 2010). Florivory can reduce the attractiveness of flowers by altering the quality and quantity of diverse floral traits, such as petal size or nectar production (McCall & Irwin, 2006; McCall, 2008; Cardel & Koptur, 2010). Florivory can also critically damage floral structures that are important for fruit and seed development (McCall, 2008; Cardel & Koptur, 2010). Visitors to flowers thus have multiple and diverse effects on plants (Kessler & Halitschke, 2009; Farré-Armengol *et al.*, 2013).

Plants have several strategies to attract pollinators to their flowers for pollination and reproductive outcrossing (Chittka & Raine, 2006; Sheehan *et al.*, 2012; Schiestl & Johnson, 2013). Plants also have mechanisms (toxins, deterrents and physical barriers) and strategies (escape in time or space) to prevent visits from visitors such as larcenists and herbivores that can have significant negative effects on fitness (Irwin *et al.*, 2004). Among these mechanisms, the emission of volatile organic compounds (VOCs) such as terpenoids, benzenoids and fatty acid derivatives is used by plants to attract or deter various visitors to flowers (Kessler *et al.*, 2008, 2013; Junker & Blüthgen, 2010; Farré-Armengol *et al.*, 2013). Benzenoids mostly function as attractants in floral scents, while floral terpenoids can both attract and deter visitors (Farré-Armengol *et al.*, 2013).

Some VOCs are instantaneously released in high amounts from damaged plant tissues. These wound-related VOCs are mostly fatty acid derivatives generically known as green leaf volatiles (GLVs) (Matsui, 2006). Herbivore-induced plant volatiles (HIPVs), especially GLVs, play a crucial role in tritrophic interactions by being involved in a mechanism of indirect defense that attracts predators and parasitoids of the herbivores (Whitman & Eller, 1990; Llusià & Peñuelas, 2001; Dicke, 2009; Hopkins *et al.*, 2009). HIPVs also mediate plant-to-plant communication by inducing defensive responses against herbivores in neighboring undamaged plants or in undamaged tissues of the same plant (Blande *et al.*, 2010; Rodriguez-Saona & Frost, 2010; Seco *et al.*, 2011; Heil, 2014).

The emission of HIPVs by flowers may indiscriminately deter both pollinators and florivores and thus interfere with pollination (Dicke & Baldwin, 2010). Herbivory could thus have major detrimental effects on plant fitness when HIPVs are emitted by attacked flowers but also when the systemic transduction of defensive chemical responses is induced from damaged leaves or flowers to undamaged flowers (Lucas-Barbosa *et al.*, 2011). Few studies, however, have demonstrated the induction of defensive VOCs in flowers in response to florivory (Muhlemann *et al.*, 2014) or to the interaction between folivory and florivory.

We characterized the floral VOC emissions of *Diplotaxis erucoides* subjected to folivory and florivory by *Pieris brassicae* caterpillars. We hypothesized that folivory and florivory could induce the emission of floral HIPVs and that florivory would immediately induce the emission of GLVs. We thus compared the floral VOC emissions from plants subjected to florivory and folivory. Most herbivores feed on both flowers and leaves, so plants infested by herbivores are expected to experience folivory and florivory at the same time (when in flower). We thus also subjected plants to a combined treatment of both folivory and florivory to test for additive or synergistic effects.

Materials and Methods

Experimental Design of Bioassays

Twenty *D. erucoides* plants were collected near Cerdanyola del Vallès (Barcelona, Catalonia, NE Spain) and were transplanted in pots. We tested four treatments, each with five plants: a control, folivory, florivory and folivory+florivory. VOCs were measured once in the morning (8:00-12:00) from each plant in each treatment before caterpillars were applied and four times once the caterpillars started to feed on the flowers and leaves. The first post-treatment measurement was immediately after applying starved *P. brassicae* caterpillars (all treatments except the control) and verifying that they began to eat leaves and/or flowers. The second post-treatment measurement was on the same day in the afternoon (14:00-17:00), and the third and fourth post-treatment measurements were on the following morning (8:00-11:00) and afternoon (12:00-15:00), respectively. The caterpillars were allowed to feed on the plants continuously during the two days of measurement.

The *P. brassicae* caterpillars had been captured from the field at the 1st and 2nd instar stages. They were fed on *D. erucoides* plants until the 3rd instar stage when they begin to feed more and cause significant amounts of damage to their host plants and begin to show a preference for plant tissues other than leaves, such as flowers, which present more attractive nutritional properties (Smallegange *et al.*, 2007). We applied caterpillars from the 3rd to the 5th (last) instar to the *D. erucoides* plants to feed on the flowers and/or leaves, depending on the treatment. The caterpillars were starved for two hours before application to ensure that they would begin to feed immediately. Five caterpillars were applied to basal leaves in the folivory

treatment, and two caterpillars were applied to an inflorescence in the florivory treatment. Seven caterpillars, two on an inflorescence and five on the basal leaves, were applied in the florivory+folivory treatment. We controlled the location of the caterpillars by enclosing the inflorescences in gauze bags or by preventing access to flowers.

We used a portable infrared gas analyzer (IRGA) system (LC-Pro+, ADC BioScientific Ltd., Herts, England) with a conifer leaf chamber (175 cm^3) to sample floral VOC emissions at standard conditions of temperature (30°C) and light (PAR=1000 µmol m^{-2} s^{-1}) and periodically measured CO_2 and H_2O exchange. An inflorescence containing 4-11 open flowers was enclosed in the chamber without detaching the flowers from the plant. For samples in the florivory and folivory+florivory treatments, we put the inflorescences with the caterpillars in the chamber and recorded the times at which the caterpillars began to feed for detecting and measuring floral GLVs instantaneously released by wounded floral tissues. We also measured several blank samples containing only caterpillars to identify possible caterpillar emissions and to distinguish them from the floral emissions.

Biogenic VOC (BVOC) Exchange Measurements

Floral CO_2 and H_2O exchanges were measured with an LC-Pro+ Photosynthesis System (ADC BioScientific Ltd., Herts, England). Flow meters monitored the air flowing through the LC-Pro+ chamber to determine and quantify BVOC exchange, and the air exiting the chamber was analyzed by proton transfer reaction-mass spectrometry (PTR-MS; Ionicon Analytik, Innsbruck, Austria). The floral terpene emissions were calculated from the difference between the concentrations of terpenes passing through the chamber clamped to the flowers and the chamber without flowers, together with the flow rates. The leaf chamber was connected to the PTR-MS system using a Teflon® tube (50 cm long and 2 mm internal diameter). The system was identical for all measurements in all treatments and blanks.

PTR-MS is based on chemical ionization, specifically non-dissociative proton transfer from H_3O^+ ions to most of the common BVOCs and has been fully described elsewhere (Peñuelas *et al.*, 2005). The PTR-MS drift tube was operated at 2.1 mbar and 50˚C, with an E/N (electric field/molecule number density) of approximately 130 Td (townsend) (1 Td = 10^{-17} V cm^2). The primary ion signal (H_3O^+) was maintained at approximately 6 × 10^6 counts per second. The instrument was calibrated with a mixed aromatic standard gas (TO-14A, Restek, Bellefonte, USA) and a monoterpene standard gas (Abello Linde SA, Barcelona, Spain).

Statistical Analyses

We conducted analyses of variance (ANOVAs) with R software (R Development Core Team, 2011) to test the differences between pre- and post-treatment measurements for each compound and treatment. We conducted t-tests with STATISTICA 8 to analyze if relative increases in floral emission rates were significantly higher than 1.

Results

The feeding by *P. brassicae* caterpillars on floral tissues produced immediate and radical changes in floral emission rates (Figure 1). The rates of emission of masses 33 (methanol), 68 (likely 3-butenenitrile) and 89 (ethyl acetate) increased immediately in the florivory and folivory+florivory treatments (Figure 1). The peaks of masses 68 and 89 fluctuated highly on a

short timescale. The emissions of mass 33 were more constant and continuous after the initial increase compared to masses 68 and 89.

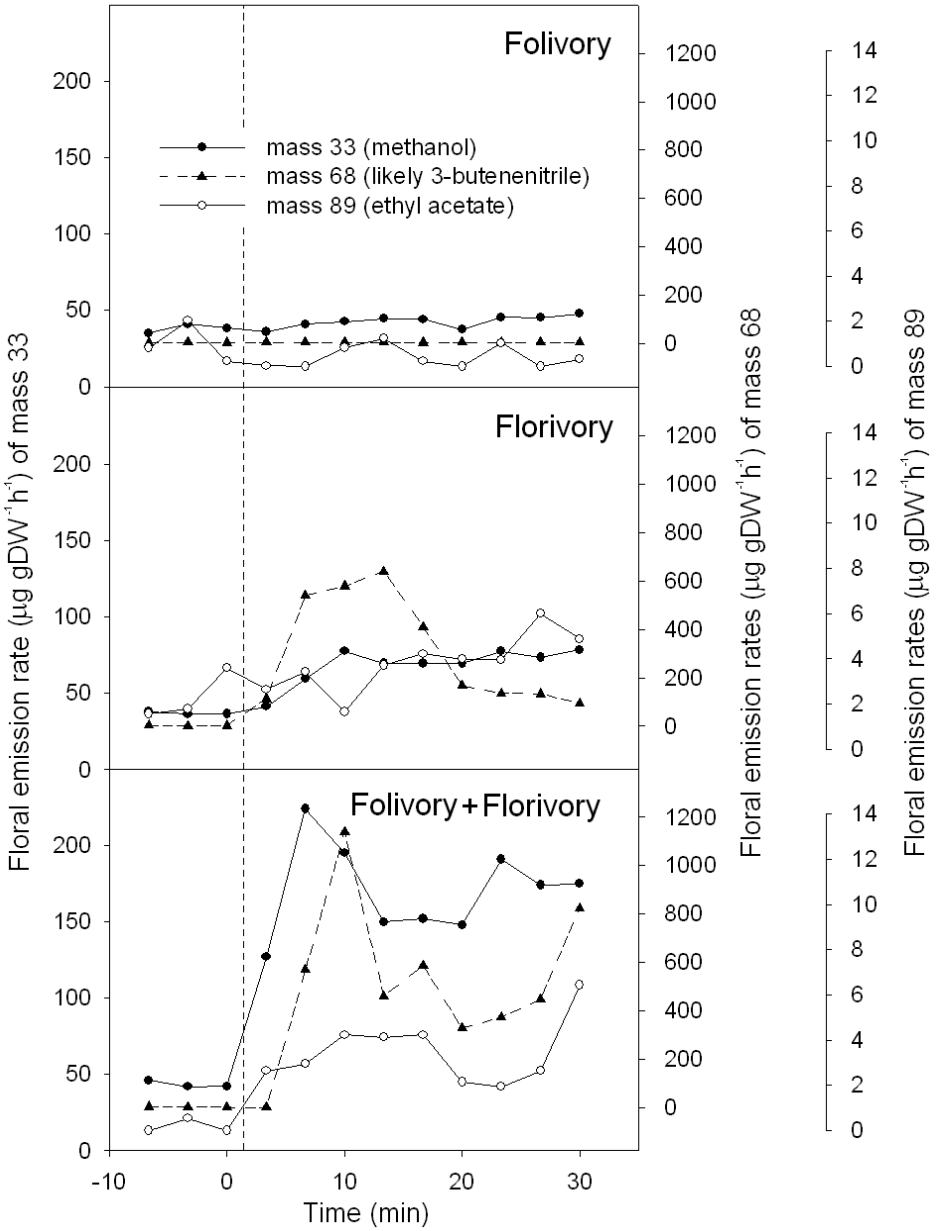

Figure 1. Dynamics of floral emission rates of masses 33 (methanol), 68 (likely 3-butenenitrile) and 89 (ethyl acetate) from one individual of each treatment on a short timescale before and after herbivorous attack.

The floral emissions of the measured masses did not change significantly in the folivory treatment relative to the control treatment throughout the monitored period (Figure 2). The emission rates of masses 33, 68 and 89 from the flowers of the plants increased 2.4- (P=0.055), 26- (P=0.099) and 2.8-fold (P=0.38), respectively, in the florivory treatment and 2.9- (P=0.009), 100- (P=0.047) and 9-fold (P=0.025), respectively, in the folivory+florivory treatment relative to the control treatment (Figure 3).

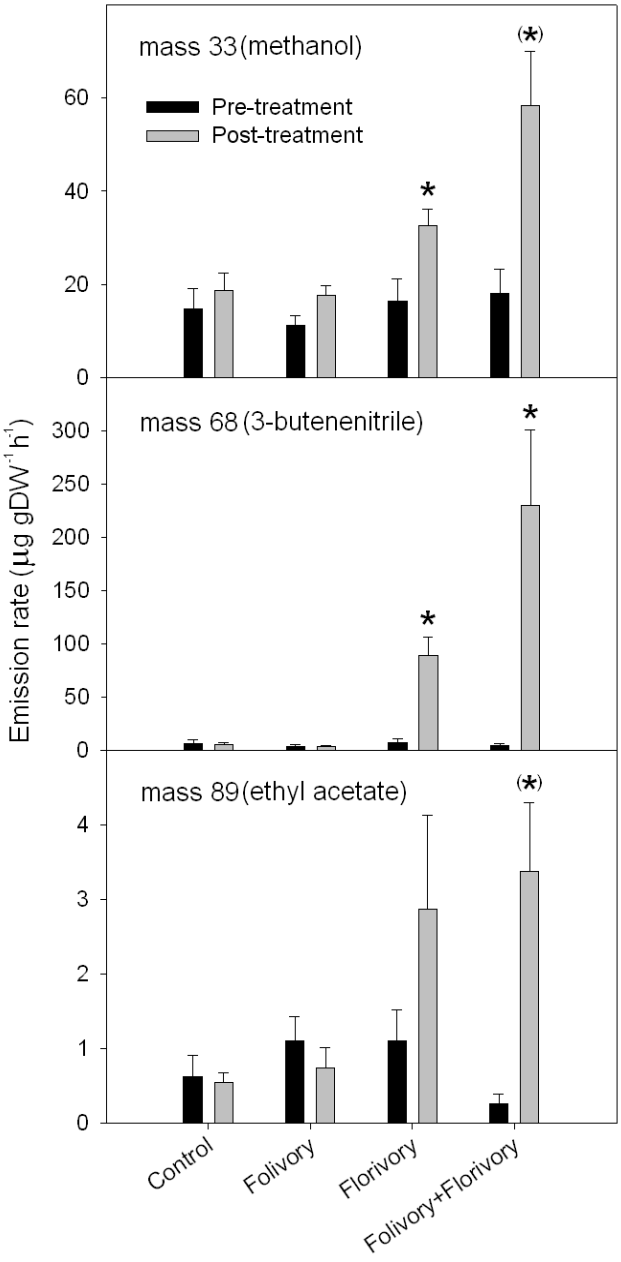

Figure 2. Mean floral emission rates of masses 33 (methanol), 68 (likely 3-butenenitrile) and 89 (ethyl acetate) before and after treatment application (n=5 plants). Error bars indicate standard errors of the means. Asterisks indicate significant differences between pre- and post-treatment measurements ($^{(*)}$ P<0.1, * P<0.05).

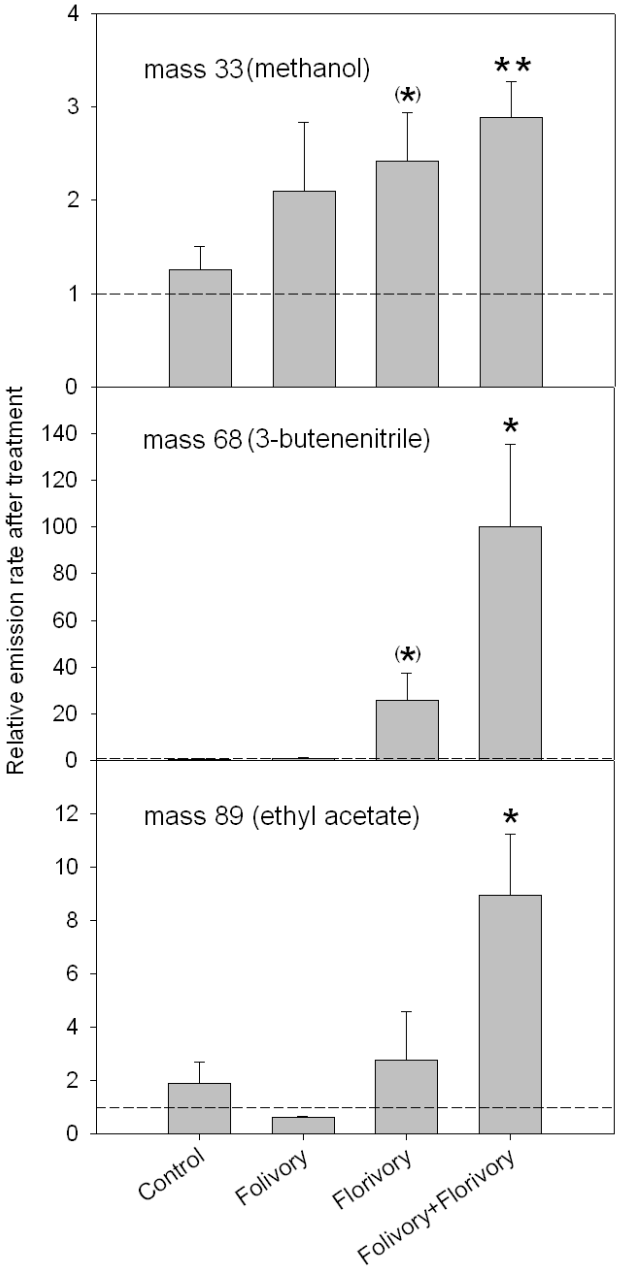

Figure 3. Mean relative increase (relative to 1, dotted lines) in floral emission rates of masses 33 (methanol), 68 (likely 3-butenenitrile) and 89 (ethyl acetate) after treatment (n=5 plants). Error bars indicate standard errors of the means. Asterisks indicate statistically significant relative increases (t-test, $^{(*)}$ P<0.1, * P<0.05, ** P<0.01).

Discussion

Floral Volatiles Enhanced by Folivory and Florivory

The emission rates of masses 33, 68 and 89 did not increase significantly in the folivory treatment, increased only marginally significantly in the florivory treatment but increased significantly in the folivory+florivory treatment (Figure 2). Only methanol has been detected with PTR-MS at mass 33 (Warneke *et al.*, 2003, 2011). The protonated mass 68 detected by PTR-MS is very likely a glucosinolate derivative, such as 3-butenenitrile (molar mass 67). Glucosinolates are a group of chemicals typical in plants of the family Brassicaceae and are usually released after tissue damage, especially due to herbivorous attack (Tsao *et al.*, 2002). Mass 89 is the primary PTR-MS mass for ethyl acetate (Steeghs *et al.*, 2004). The emission rates of mass 89 have also been correlated with those of masses 61 and 71, which are secondary masses of ethyl acetate (Steeghs *et al.*, 2004).

Florivory caused an immediate increase in the emission rates of masses 33 (methanol), 68 (likely 3-butenenitrile) and 89 (ethyl acetate) in both the florivory and folivory+florivory treatments (Figure 1). All these compounds are released in high amounts immediately after damage to plant tissues. Methanol is a ubiquitous and well-known VOC that is normally emitted at high rates by undamaged plants but is also locally released in high amounts by wounded tissues (Peñuelas *et al.*, 2005). Methanol is produced from pectin demethylation in the cell walls (Galbally & Kirstine, 2002; Seco *et al.*, 2007), so significant methanol emissions are expected from damaged plant tissues because pectin demethylation occurs in the apoplast, and methanol is a common constituent of the transpiratory stream in plants (Fall & Benson, 1996). Additionally, alkaline oral secretions from lepidopteran larvae induce a change in pH at the wound site that can strongly enhance methanol emissions (von Dahl *et al.*, 2006). The compound emitted most by flowers subjected to florivory, 3-butenenitrile, is a glucosinolate derivative and thus has insecticidal activity in plants attacked by herbivores (Tsao *et al.*, 2002). Some degradation products of glucosinolates, such as isothiocyanates, nitriles and thiocyanates, also participate in the induction of stomatal closure after herbivorous attack, suggesting that these degradation products regulate stomatal movements against attacks by phytophagous insects (Hossain *et al.*, 2013). Ethyl acetate is emitted by some plant species in response to herbivorous and pathogenic attack from various plant structures, such as leaves (Zhang *et al.*, 2008), roots (Steeghs *et al.*, 2004) and fruits (Benelli *et al.*, 2013).

Dynamic Response of Floral Emissions to Florivory

Floral emissions increased quickly in response to the attack on flowers by *P. brassicae* caterpillars (Figure 1) but did not change significantly in the final 28 h of the treatments. This immediate response indicated that the VOCs in the flowers were released from the wounded tissues once the caterpillars had begun to feed. The floral emission rates of masses 68 and 89 fluctuated highly on a short timescale (Figure 1), which may indicate a very fast response of these compounds to the dynamic fluctuations in the intensity of the damage caused by the feeding *P. brassicae* caterpillars. The emission rates of mass 33, however, were more constant after the initial increase in response to attack. An increase in methanol emissions by wounded plant tissues can be mostly due to the direct release from internal tissues after damage (Peñuelas *et al.*, 2005).

Herbivore-Induced Plant Volatiles and Systemic Defensive Responses

Defensive compounds can deter both detrimental and beneficial visitors to flowers in a similar way. The constitutive emission of repellent compounds to deter herbivores can thus imply disadvantages to plant fitness by the interference of pollination, which can sometimes exceed the benefits of avoiding enemies (Lucas-Barbosa *et al.*, 2011). Selective pressures may then reduce or eliminate such deterrent compounds from floral emissions, due to the negative impact they have on plant fitness. From this viewpoint, plants may benefit from presenting defenses that are activated only when necessary, such as the HIPVs emitted after herbivorous attack. Induced defensive responses provide other benefits to plants compared to constitutive defenses, such as their activation only when needed, representing a more optimal investment of resources for defense (Pare & Tumlinson, 1999).

The induced emission of HIPVs during the flowering season, however, can imply detrimental effects on plant pollination (Lucas-Barbosa *et al.*, 2011). The emission of HIPVs can be systemically induced from damaged to undamaged leaves (Rodriguez-Saona *et al.*, 2009; Dong *et al.*, 2011) and to undamaged flowers (Kessler & Halitschke, 2009; Theis *et al.*, 2009). This systemic induction of deterrent emissions from damaged to undamaged plant tissues can also interfere with the attraction of pollinators, but some species can avoid the induction of HIPVs when they can interfere with pollinator attraction. HIPV emissions from *Datura wrightii*, for example, are high during the vegetative phase but decline after the beginning of flowering and fruit production (Hare, 2010). This timing may avoid the counterproductive effect of HIPVs on pollinator visits.

We found no evidence for a systemic induction of defensive floral VOC emissions in response to folivory in *D. erucoides*. Folivory combined with florivory, however, increased floral VOC emissions, perhaps by inducing a synergistic systemic effect. *Diplotaxis erucoides* plants grow quickly and flower early and for a substantial portion of their lives. The long flowering period may have generated selection pressures to suppress herbivory-induced systemic responses in this species to avoid interference with pollinator attraction. Florivory caused only a local immediate increase in the emission rates of some volatiles in flowers damaged by *P. brassicae* caterpillars. This local defensive response may only deter herbivores temporarily at the site of damage so may not interfere with the pollination of distant undamaged flowers that are still attractive and viable. Similarly, *Nicotiana suaveolens* plants subjected to green-leaf herbivory emitted HIPVs from leaves but not from flowers, suggesting that the response to herbivory was systemic among leaves but was not transmitted to flowers (Effmert *et al.*, 2008). In fact, flowers can show no induction of enhanced floral emissions in response to folivory and can even reduce their emissions due to tradeoffs between pollinator attraction and indirect defenses induced in other plant tissues (Schiestl *et al.*, 2014).

Synergistic Effect of the Folivory+Florivory Treatment

Folivory alone had no clear significant effects on the emissions rates of floral volatiles. A synergistic effect on the emission rates of floral VOCs, however, was evident when folivory was combined with florivory. The relative increases in the emission rates of masses 33, 68 and 89 between pre and post-treatment were 1.2-, 4- and 3-fold higher, respectively, in the plants subjected to the combined treatment than in the plants subjected only to florivory (Figure 3).

All these results strongly suggest a synergistic effect of folivory and florivory. Such an effect may intensify the magnitude of the chemical defensive response when both flowers and leaves are attacked, which usually indicates a wider degree of infestation. Plants may benefit

from increasing their defenses when herbivorous attack is more severe and generalized compared to mild and local attacks. These results are the first reported indication of a synergistic effect of folivory and florivory on floral emissions.

Conclusions

Floral emissions of masses 33 (methanol), 68 (likely 3-butenenitrile) and 89 (ethyl acetate) increased immediately and significantly when *P. brassicae* caterpillars began to feed on flowers but did not show delayed induced responses to florivory. The responses or changes in floral emissions were not gradual, apart from the increases in those compounds emitted immediately after the feeding of caterpillars on flowers and that persisted throughout the period of the treatments. Systemic induction of volatile emissions from damaged leaves to intact flowers was not observed in the plants subjected to folivory. VOC emission rates nevertheless increased more in the combined treatment of folivory and florivory than in the florivory treatment alone, indicating a synergistic effect of folivory and florivory that intensified the floral defensive response when the attack was more generalized to the entire plant.

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Chapter 7. Changes in floral bouquets from compound-specific responses to increasing temperatures

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Abstract

We addressed the potential effects of changes in ambient temperature on the profiles of volatile emissions from flowers and tested whether warming could induce significant quantitative and qualitative changes in floral emissions, which would potentially interfere with plant–pollinator chemical communication. We measured the temperature responses of floral emissions of various common species of Mediterranean plants using dynamic headspace sampling and used GC-MS to identify and quantify the emitted terpenes. Floral emissions increased with temperature to an optimum and thereafter decreased. The responses to temperature modeled here predicted increases in the rates of floral terpene emission of 0.03–1.4-fold, depending on the species, in response to an increase of 1°C in the mean global ambient temperature. Under the warmest projections that predict a maximum increase of 5°C in the mean temperature of Mediterranean climates in the Northern Hemisphere by the end of the century, our models predicted increases in the rates of floral terpene emissions of 0.34–9.1-fold, depending on the species. The species with the lowest emission rates had the highest relative increases in floral terpene emissions with temperature increases of 1–5°C. The response of floral emissions to temperature differed among species and among different compounds within the species. Warming not only increased the rates of total emissions, but also changed the ratios among compounds that constituted the floral scents, i.e. increased the signal for pollinators, but also importantly altered the signal fidelity and probability of identification by pollinators, especially for specialists with a strong reliance on species-specific floral blends.

Keywords: chemical communication, emission profiles, flower physiology, flower volatile emissions, global warming, monoterpenes, physicochemical properties, sesquiterpenes, temperature–response curve, volatility.

Introduction

Plants use biogenic volatile organic compounds (BVOCs) to interact with both beneficial (pollinators, seed dispersers, and carnivores) and detrimental (herbivores, parasites, and competitors) organisms (Dudareva *et al.*, 2006; Fineschi *et al.*, 2013; Trowbridge & Stoy, 2013). Floral blends of volatiles constitute private communication channels between emitter plants and those animal receivers to which the volatiles are directed (Raguso, 2008). Constitutively emitted BVOCs become specific signatures that allow organisms to identify the plant species and the tissue from which the scents are emitted. BVOCs may serve, for example, to promote reproductive isolation among compatible, sympatric, closely related species by providing pollinators with distinguishable floral scents (Füssel *et al.*, 2007). Plants present a diverse array of volatile compounds to attract pollinators to their flowers for assuring pollination (Knudsen *et al.*, 2006), and pollinators use the scent trails of floral emissions to locate flowers (Cardé & Willis, 2008). Mixtures of floral BVOCs allow pollinators to identify the plant species emitting the scent and provide diverse information about the flowers, such as their developmental stage (Mactavish & Menary, 1997; Proffit *et al.*, 2008; Goodrich & Raguso, 2009) and the availability and quality of their rewards (Howell & Alarcón, 2007; Wright *et al.*, 2009). In many cases, floral chemical messages directed at pollinators contain specific mixtures of compounds with specific ratios of each emitted volatile (Raguso, 2008).

Environmental conditions can affect BVOC emissions from plants. In particular, temperature is an abiotic factor that strongly affects plant emissions (Peñuelas, 2008; Peñuelas & Staudt, 2010; Grote *et al.*, 2013). Temperature can affect emissions in two ways: first, through its effects on the physicochemical properties of BVOCs, such as volatility, solubility,

and diffusivity; and second, by affecting various plant physiological traits that play a role in some of the phases of BVOC emission, e.g., biosynthesis of BVOCs, stomatal resistance or regulated processes of release (Niinemets *et al.*, 2004). The effect of temperature on physicochemical properties is clearer than the effect on plant physiology, which depends on the species (Kesselmeier & Staudt, 1999), the effects of past and present stresses on the physiological state of a plant (Fortunati *et al.*, 2008; Niinemets, 2010) and environmental conditions such as temperature and light that modify the rate of BVOC synthesis (Penuelas & Llusia, 2001; Niinemets *et al.*, 2010a). Higher temperatures enhance the activities of enzymes involved in BVOC biosynthesis, reduce BVOC solubilities and increase BVOC volatilities (vapor pressure and partitioning to the gas phase) and diffusivities along cellular phases and thereby decrease the resistance of emission pathways, thus promoting an increase in the rates of emission (Niinemets *et al.*, 2004; Harley, 2013). Different compounds have different chemical properties and volatilities, which affect the rate of release from internal tissues. Compounds with higher volatilities will be more rapidly released, while those with lower volatilities will need to accumulate in higher amounts in intratissular nonspecific storage pools and reach higher internal concentrations to be released at similar rates (Niinemets *et al.*, 2004; Noe *et al.*, 2006).

Environmental conditions are changing globally due to human activities, and the main drivers of global change are likely to increase emissions of BVOCs by plants (Peñuelas & Staudt, 2010). The mean surface temperature in the Mediterranean Basin is projected to increase by approximately 1–5°C by the end of the century relative to the period 1850–1900 (IPCC, 2013). A temperature increase in this magnitude will induce several effects on the physiology and physicochemistry of living organisms. The rate of the current warming will exceed the ability of most plant populations and species to migrate (Neilson *et al.*, 2005), so they will not be able to move toward cooler areas to counteract the effects of global warming. Plants will thus inevitably be submitted to warmer temperatures that will cause various physiological changes and unavoidable derived effects on various functions.

The volatility of each compound has a compound-specific dependence on temperature (Llusia & Penuelas, 2000; Copolovici & Niinemets, 2005; Copolovici *et al.*, 2005). Warming may therefore not only induce a general increase in BVOC emissions, it may also induce differential changes in the rates of compound emissions due to differences in the physicochemical properties of the compounds (Niinemets & Reichstein, 2002; Noe et al., 2006) and may therefore affect the ratios of the compounds in the floral blends (Niinemets & Reichstein, 2002). Staudt & Bertin (1998) observed significant changes in the relative composition of terpenes in the foliar emissions from *Quercus ilex* along a temperature gradient of 5–45°C. Major changes in the emission profile were due to a stronger response of the acyclic monoterpenes cis- and trans-β-ocimene from 35 to 45°C, compared to that of mono- and bicyclic monoterpenes that stabilized near 35°C, and to the induction of sesquiterpene caryophyllene emissions (Staudt & Bertin, 1998). Induced emissions due to heat stress at extreme temperatures (Joó *et al.*, 2011; Copolovici *et al.*, 2012) may also induce qualitative changes in floral scents. All these changes in the amount and relative composition of plant emissions can affect the correct establishment of specific communication channels between plants and mutualists.

Changes in temperature and other accompanying factors associated with global change are thus expected to induce quantitative and qualitative changes in floral BVOC emissions (Peñuelas, 2008; Peñuelas & Staudt, 2010) that could affect plant–pollinator interactions in several ways (Farré-Armengol *et al.*, 2013). Our goals were to assess the effects of warming on floral emissions and to test our hypothesis that increases in ambient temperature would induce quantitative and qualitative variations in floral terpene emissions.

We also quantified these variations in seven widespread species of Mediterranean plants with differing flowering phenologies.

Materials and methods

Measurement of temperature responses

Seven common Mediterranean species [*Globularia alypum* (L.) Greuter, *Erica multiflora* L., *Q. ilex* L., *Dorycnium pentaphyllum* Scop., *Spartium junceum* L., *Sonchus tenerrimus* L., and *Dittrichia viscosa* L.] growing in the field were selected for the experiments. The plants were chosen from various locations in the province of Barcelona (Catalonia, Spain). We chose the species taking into consideration their commonness and ecological representativeness. We chose species that flower at different seasons of the year: *G. alypum* and *Erica multiflora* flowered in winter, *Q. ilex* and *D. pentaphyllum* in spring, *S. junceum* and *S. tenerrimus* in summer, and *D. viscosa* from late summer to early autumn. In addition, *Q. ilex* was chosen as a model of a typical anemophilous species. The measurements were conducted at periods of peak flowering, except for *D. viscosa* that was tested both in late summer and again in early autumn at the end of the flowering period. The experimental setup for the winter-flowering species *G. alypum* and *E. multiflora* only allowed for the measurement of temperature responses to 30°C. In all other cases, the temperature responses were measured over a temperature range of 15–40°C, at intervals of 5°C. We measured three to six replicate temperature responses per species, and the response of each replicate was measured from a different plant.

Samples were collected under field conditions using a dynamic headspace technique. We employed a portable infrared gas analyzer (IRGA) system (LC-Pro+, ADC BioScientific Ltd., Great Amwell, UK) to create the required conditions of temperature and to provide a constant light intensity of 1000 µmol m^{-2} s^{-1} for the sample tissue and to record periodic measurements of variables of gas exchange. One or several attached flowers for each sample were enclosed in the chamber of the IRGA. We used either a broad leaf chamber (12 cm^3) or a conifer leaf chamber (175 cm^3), depending on the size of the flowers of each species (but always the same size of chamber for all samples from each species). We collected the samples of terpene emissions after setting the required quantum flux density and temperature and after an acclimation period of approximately 10 min or the time needed to reach a steady-state exchange of CO_2 and H_2O. The air exiting the leaf cuvette, with a mean flux of air of approximately 200–250 ml min^{-1}, was directed through a Teflon tube to a tube filled with the adsorbents Tenax (50% vol.) and Carbotrap (50% vol.), which collected the terpenes emitted by the flower(s) over a period of 10–15 min. The same process was repeated with empty leaf cuvettes that served as blanks of the system. At least two blank samples were collected for each curve, one at the beginning of the emission samplings and another at the end. After each sampling sequence, we collected the flower samples to dry and weigh them for emission rate calculations.

Terpene analyses

The terpene samples in the adsorbent tubes were thermally desorbed, and the samples were analyzed by gas chromatography-mass spectrometry (GC-MS; GC: 7890A, MS: 5975C inert mass spectrometric detector with Triple-Axis Detector, Agilent Technologies, Palo Alto, CA, USA). Samples were injected into a 30 m 9 0.25 mm 9 0.25 lm capillary column (HP-5MS; Agilent Technologies). Helium flow was 1 ml min^{-1}, and total run time was 26 min. After

injection, the sample was maintained for 1 min at 35°C, the temperature was then increased at 15°C min^{-1} –150°C and maintained for 5 min, then increased at 50°C min^{-1} –250°C and maintained for 5 min and then increased at 30°C min^{-1} –280°C and maintained for 5 min.

The terpenes were identified by comparing the retention times with standards (Fluka, Buchs, Switzerland) that had been injected into clean adsorbent tubes, and the fractionation mass spectra were compared with standard spectra and spectra in the Nist05a and wiley7n mass spectral libraries. Calibration curves for the common terpenes α-pinene, β-pinene, D-limonene, γ-terpinene, linalool and α-humulene were determined each day of the analysis. The terpene calibration curves (n = 4 different terpene concentrations) were always highly significant (r2 > 0.99 for the relationship between the signal and the amount of compound injected). Terpene concentrations were determined from the calibration curves.

Statistical treatment

We used the lme function of the nlme package of the R software (Pinheiro *et al.*, 2013) to analyze the changes in the relative percentage ratios of the terpene compounds along temperature gradients. We considered plant individuals as a random factor in the analysis. The temperature–response curves of floral terpene emissions were fitted by local polynomial functions using the loess function of R (Cleveland *et al.*, 1992; R Development Core Team, 2011). The fitted models were used to calculate the predicted emission rates of floral terpenes at the mean maximum temperature of the month of the flowering peak of the species at the sampling location (T_{peak}). Thereafter, we used the fitted models to predict the emission rates at temperatures of 1, 2, 3, 4 and 5°C above T_{peak} to explore the potential changes in floral terpene emissions in response to the temperature increases projected for the coming decades by global circulation models (IPCC, 2013).

Results

Total terpene emissions

The rates of terpene emissions initially increased with temperature in all species to an optimum temperature and decreased in most species at higher temperatures (Figure 1). The temperature responses varied, depending on species and the spectrum of compounds emitted. The flowers of *G. alypum* and *E. multiflora* had maximum terpene emissions at 25–30°C. *Quercus ilex* floral emission rates reached a maximum at approximately 30°C. The rates of terpene emission from the flowers of *D. pentaphyllum* increased with temperature up to 35°C and decreased slightly at 40°C. The rates of terpene emission from the flowers of *D. viscosa*, *S. junceum*, and *S. tenerrimus* increased with temperature even at the highest tested temperature of 40°C, and the maximum increase was observed between temperatures of 30–40°C. The measurements conducted on *D. viscosa* in early autumn, however, changed considerably compared with those in late summer. The emission maxima in early autumn occurred at 25–30°C, while the maxima in late summer occurred at 40°C or higher.

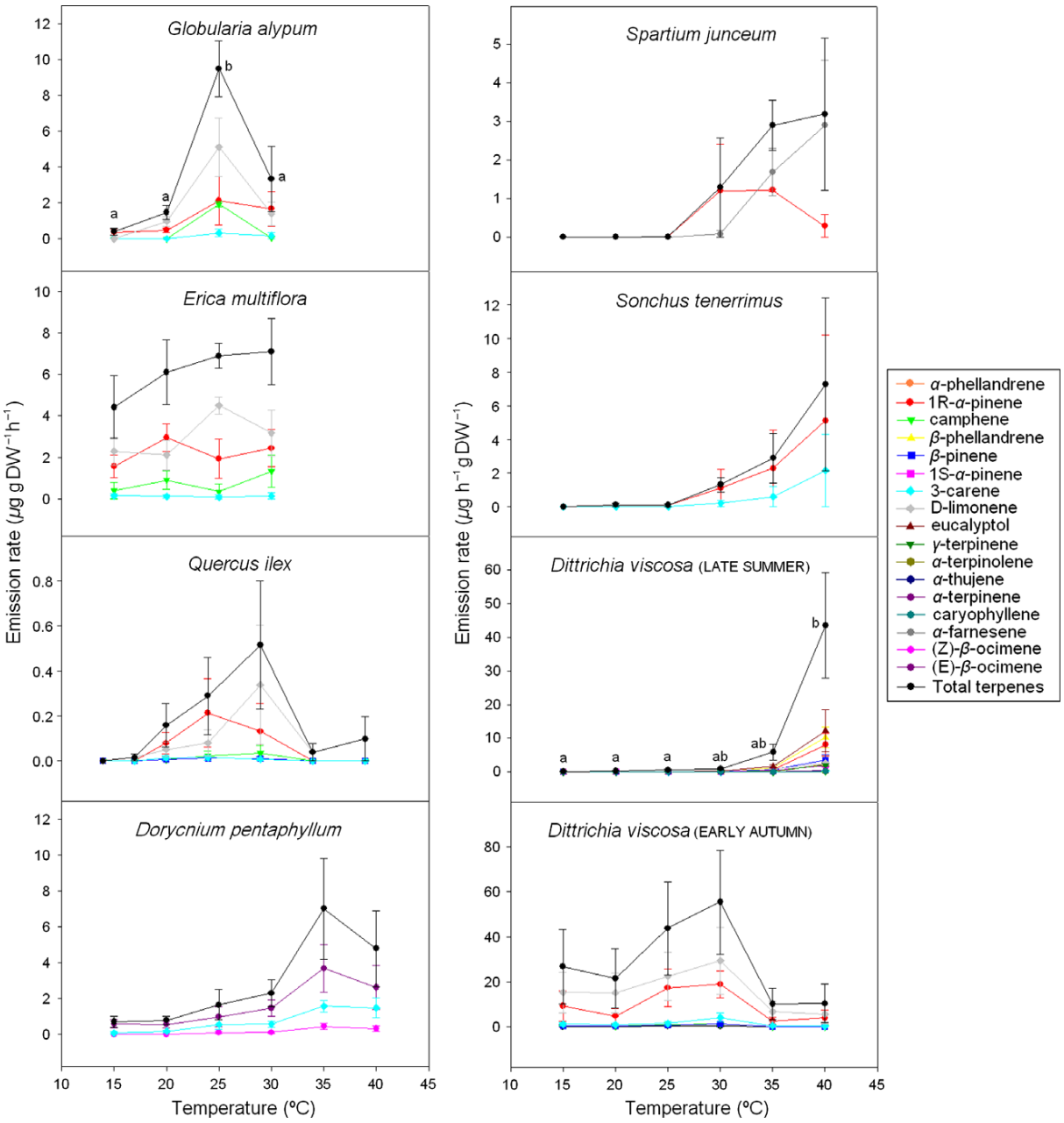

Figure 1. Emission rates (lg gDW^{-1} h^{-1}) of single and total terpenes from the flowers of seven Mediterranean species over a temperature gradient of 15–40 °C. The quantum flux density was maintained at 1000 lmol m^{-2} s^{-1} during the measurements. Error bars indicate SE (n = 3–6). Letters indicate significant differences among the emission rates at different temperatures.

Relative terpene composition of floral scents

The relative composition of floral emissions varied with temperature (Figure 2). Only some compounds in some species, however, displayed significant trends in the relative abundance along temperature gradients. D-limonene, which was predominant at low temperatures, was partially substituted by 1R-α-pinene at higher temperatures in the floral scent of *G. alypum*. The patterns of decrease and increase in the relative abundances of D-limonene and 1R-α-pinene, however, were not statistically significant. Terpene ratios in the floral scent of *E. multiflora* did not change significantly with temperature. The floral emissions from *Q. ilex* were entirely composed of D-limonene at high temperatures (35 and 40°C) and contained other

monoterpenes from 20 to 30°C, but the percentages of each compound followed no significant trends. The relative composition of terpenes in the floral scent of *D. pentaphyllum* showed a gradual substitution of 3-carene, which experienced a reduction in its relative percentage ($P < 0.001$) with increasing temperature, by the two isomers (E)- and (Z)-β-ocimene that increased their relative abundances ($P < 0.001$ and $P = 0.001$, respectively). In the floral scent of *S. junceum*, the monoterpene 1R-α-pinene was gradually substituted by the sesquiterpene α-farnesene as temperature increased, but the trends were not significant. The floral scent of S. tenerrimus was entirely composed at low temperatures of 1R-α-pinene, which decreased at higher temperatures ($P = 0.07$) when levels of 3-carene increased ($P = 0.07$).

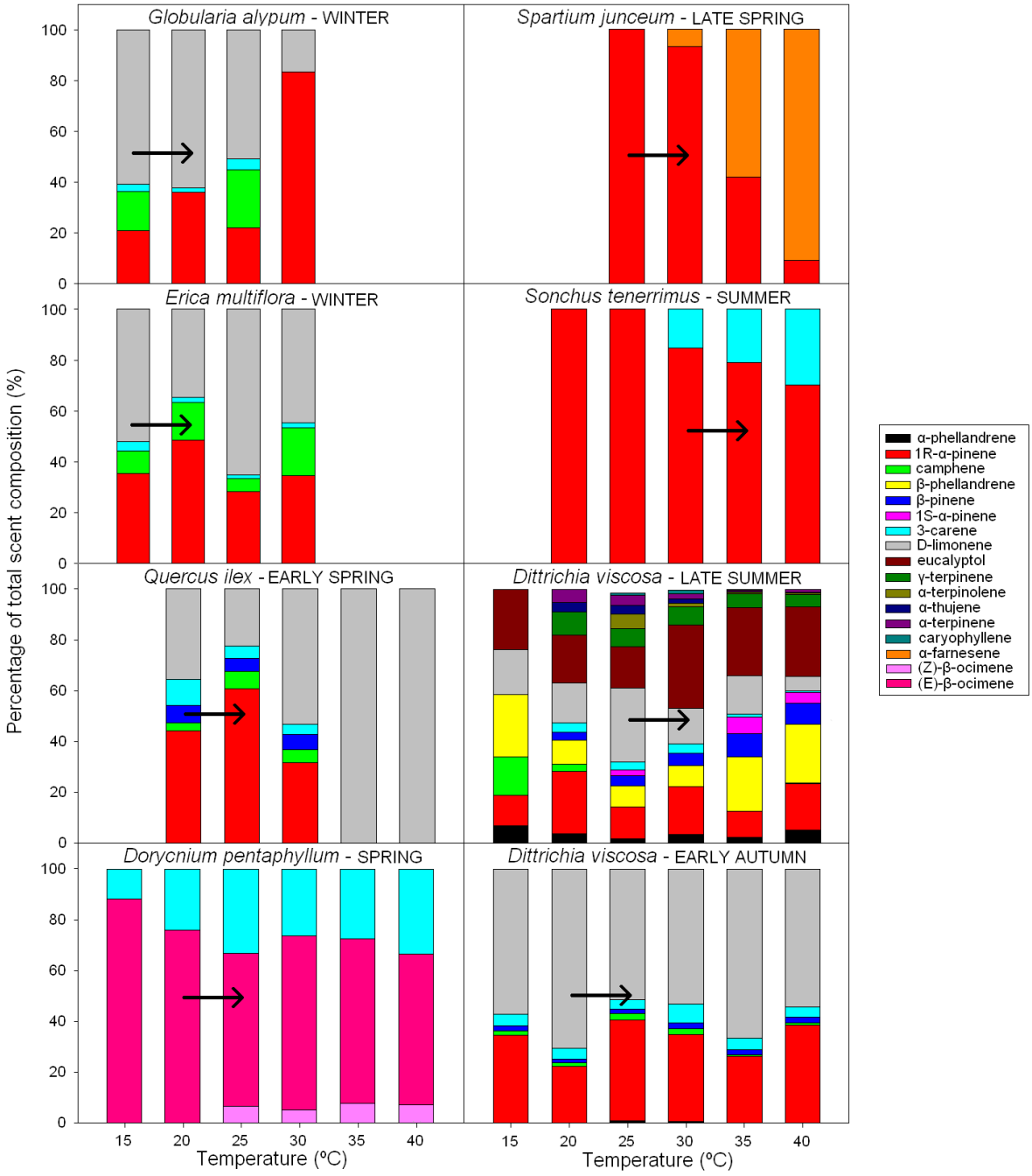

Figure 2. Ratios (%) of the rates of floral terpene emissions relative to the rates of total terpene emissions of each species over a temperature gradient of 15–40 °C. Arrows indicate the hypothetical change (assuming no acclimation of emission profiles) in the composition of floral scents when the mean maximum temperature of the flowering period of each species was increased by 5 °C, the maximum increase projected by IPCC (2013) by the end of the century.

The floral emissions from *D. viscosa* in late summer increased significantly with temperature and also changed in the relative composition of terpenes along the temperature gradient. β-pinene (P = 0.004) and 1S-α-pinene (P = 0.01) increased steadily in relative ratio, while D-limonene (P = 0.07) and camphene (P = 0.09) decreased. The relative composition of terpenes in the floral emissions from *D. viscosa* in early autumn did not vary significantly in the temperature–response curves, and the diversity of emitted volatiles was lower than in late summer. The emissions in early autumn particularly lacked eucalyptol and β-phellandrene, which were the most abundant terpenes in the floral scent of *D. viscosa* in late summer.

Predicted changes in total and relative floral emissions of terpenes with future warming

The magnitude of the stimulating effect of temperature on total emissions of floral terpenes varied among species. The modeled rates of floral terpene emission would increase 0.03–1.4-fold with a 1°C increase in mean maximum temperature, depending on the species (Table 1). Under the warmest scenario projected by the IPCC (2013), which predicts a maximum increase of 5°C in mean maximum temperatures for this century in the Mediterranean climates of the Northern Hemisphere, rates of floral terpene emissions would increase 0.34–9.1-fold. Under global warming ranging from +1 to +5°C, *S. junceum* and *Q. ilex* would have the highest relative increases in the rates of floral terpene emissions (1.4–9.1- and 0.33–7-fold, respectively); *G. alypum*, *D. pentaphyllum*, and *S. tenerrimus* would have moderate relative increases (0.1–2.55-, 0.18–1.02-, and 0.34–2.22- fold, respectively) and *D. viscosa* in late summer and early autumn and *E. multiflora* would have smaller relative increases (0.10–0.69-, 0.12–0.68-, and 0.03–0.34-fold, respectively).

Species	T_{peak} (°C)	Floral emission rates (µg g DW^{-1} h^{-1})					
		Mean max T	+1°C	+2°C	+3°C	+4°C	+5°C
Globularia alypum	14.3	0.4	0.44	0.66	0.94	1.23	1.42
Erica multiflora	14.3	4.41	4.54	4.94	5.31	5.63	5.92
Quercus ilex	18.5±	0.03	0.04	0.08	0.13	0.19	0.24
Dorycnium pentaphyllum	20.5	0.84	0.99	1.18	1.38	1.56	1.7
Spartium junceum	26	0.1	0.24	0.4	0.59	0.79	1.01
Sonchus tenerrimus	29.9	1.02	1.37	1.76	2.20	2.71	3.28
Dittrichia viscosa (late summer)	23.5	0.51	0.56	0.58	0.65	0.75	0.86
Dittrichia viscosa (early autumn)	20.8	27.8	31.1	35	39.2	43	46.8

Table 1. Predicted values of floral terpene emission rates (µg g DW^{-1} h^{-1}) of the various species at the mean maximum temperature of the month of their flowering peaks (T_{peak}) and at temperatures of 1, 2, 3, 4 and 5 °C higher than T_{peak}. The values were predicted from the loess functions that fitted the measurements of floral terpene emissions at different temperatures.

Discussion

Total terpene emissions

Our results confirm a generalized pattern of increase in the rates of floral terpene emissions with temperature, especially within the temperature range of 25–35°C. The terpenes were emitted at low rates from the flowers of the anemophilous tree *Q. ilex*, as can be expected for

a species that does not need to attract pollinators. We detected, however, some ubiquitous monoterpenes, whose emission rates reached a maximum at approximately 30°C. These results support those from Hu *et al.* (2013) who found an increase in floral emissions from 10 to 30°C, followed by a decrease at 40°C. Our results are also similar to the well-characterized temperature response of BVOC emissions in leaves (Guenther *et al.*, 1999; Penuelas & Llusia, 2001; Niinemets *et al.*, 2010b; Peñuelas & Staudt, 2010).

The global pattern of increase in floral emissions with temperature may in part be due to the temperature dependencies of the physicochemical properties of terpenes and to the enzymatic activities of terpene synthases, all of which could enhance emissions with warming. In addition, the physiology of flowers may affect the responses of floral emissions to temperature. In fact, the optimum temperatures for floral emissions varied among species even though these species shared most of the main compounds in their floral scents. These variations in the optimum temperatures among species, therefore, cannot be due to differences in the physicochemical properties of specific compounds andare also not driven by compound-specific optimal temperatures. These factors lead to the assumption that species-specific traits of floral physiology play an additional and important role in determining the responses of floral emissions to temperature. Floral physiology controls the production of each compound through the regulation of transcription, production and activity of enzymes, and the concentrations of the substrates of these enzymes (Dudareva & Pichersky, 2000; van Schie *et al.*, 2006). A broad array of terpene synthases is responsible for the formation of floral volatiles (Pichersky *et al.*, 2006; Dudareva & Pichersky, 2008). Some of these synthases are highly specific, forming only one product, while others form multiple products (Dudareva *et al.*, 1996, 2003; Nagegowda *et al.*, 2008; Memari & Pazouki, 2013). In response to variable temperature conditions, floral physiology can thus modify biosynthetic activity to regulate the emission of each floral compound or of multiple compounds simultaneously, depending on synthase specificity.

Relative terpene compositions of floral scents

The magnitude of the changes to the relative composition of floral terpene blends driven by temperature also varied among species. The temperature-driven shifts observed in floral terpene composition (Figure 2) allow us to predict some compositional changes in the floral terpene blends in response to warming. The changes in relative floral terpene composition after increasing the temperature 5°C were not significant (Figure 2), but they followed the significant trends of change over the entire temperature responses described in the previous section. For *D. pentaphyllum* (20–25°C) and *S. junceum* (25–30°C), additional compounds that are not emitted at the current mean maximum temperature of the flowering period are expected to be present in floral blends in warmer climates [(Z)-β-ocimene and α-farnesene, respectively]. The floral blend of *G. alypum* (15–20°C) may not drastically change compositionally, except for the loss of camphene from the blend. The relative ratios of various compounds would change subtly in the floral terpene emissions from *E. multiflora* (15–20°C), Q. ilex (20–25°C), *S. tenerrimus* (30–35°C), and *D. viscosa* (25–30°C in late summer; 20–25°C in early autumn). Relative increases in 1R-α-pinene over D-limonene are predicted for the floral emissions of *E. multiflora* and *Q. ilex* and the *D. viscosa* plants flowering in early autumn. A relative increase in 3-carene over 1R-α-pinene is predicted for the floral blend of *S. tenerrimus*. A relative increase in eucalyptol and 1R-α-pinene over α-terpinolene and D-limonene is predicted for the floral emissions of D. viscosa plants flowering in late summer. All these compositional changes are in agreement with the findings of Hu *et al.* (2013) showing that *Lilium* 'siberia' plants emit different terpenes at different temperatures and also that the emission rates of different BVOC chemical groups (terpenes, aromatics, alkanes, aldehydes,

etc.) show different temperature–response curves, leading to scent shifts not only in terpene composition but also in BVOC chemical groups.

Seasonal variation of the temperature response

We detected intraspecific seasonal changes in the temperature responses of total terpene emissions in *D. viscosa*, which was sampled in late summer and again in autumn. We also observed a reduction in the diversity of terpene signatures constituting the floral blend in autumn. Intraspecific seasonal differences in the responses of terpene emissions to temperature have also been observed in leaves (Llusia *et al.*, 2006). These seasonal changes also point to physiology as a factor that not only determines the temperature response of floral emissions but also modulates the shape of this response at the intraspecific level, depending on the season. Such intraspecific variations demonstrate large temperature-driven plasticity of plant physiological traits and clearly emphasize the need to consider genotypic, epigenetic, and phenotypic plasticity in estimating and modeling floral emissions.

Altered floral emissions in a warmer world and implications for pollinators

Projections of mean surface temperatures in the Mediterranean Basin predict an increase of approximately 1–5°C relative to the period 1850–1900 by the end of the century (IPCC, 2013). If a direct projection is made from the temperature responses obtained here, the rates of floral terpene emission by the end of the century could increase 0.34–9.1-fold for a 5°C increase in mean maximum temperature during the flowering peak of the species (Table 1). Such a broad range of variation in the magnitude of the increase in floral terpene emissions in response to global warming points to future significant differences among species in the intensity of floral olfactive signals. The species with the highest relative increases in floral terpene emissions were those with the lowest rates of emission, so we may expect the lightly scented flowers of these species to significantly increase the intensity of their olfactive signals, while increases in the signal intensity of strongly scented species will be low to moderate.

The relative composition of terpenes along temperature curves changed significantly in the floral blends of some species, especially at the highest temperature ranges and in those species that flower in the warmest seasons. Some of the observed changes were small, while some implied substitutions of the predominant compounds in the floral blend. The expected changes in the relative terpene composition of floral scents in response to an increase in temperature of 1–5°C, which are likely to occur by the end of the century (IPCC, 2013), may imply changes to the composition of floral scents in some species (Figure 2). The changes in composition that we observed at higher temperatures included changes in the relative abundance of preexisting compounds, the appearance of new compounds or the disappearance of compounds that are emitted at current temperatures. Heat stress can cause variations in the composition of floral scents, such as the appearance or increase in some compounds only at high temperatures (Niinemets, 2010; Copolovici *et al.*, 2012).

An increase in terpene emissions in response to the predicted warmer temperatures from global warming may extend the physical range of the signals that pollinators detect and follow (Peñuelas, 2008; Peñuelas & Staudt, 2010; Niinemets *et al.*, 2013) but also implies the attraction of a broader group of pollinators with varying efficiencies of signal reception. Higher concentrations of floral scents may also increase the importance of the olfactory stimulus, thus leading to enhanced initial responses and learned performances of the pollinators (Katzenberger *et al.*, 2013).

Increased floral emissions, however, may also have negative effects. A significant increase in the emission of floral terpenes, and in terpene emissions from other tissues that also respond positively to temperature increases, may imply higher metabolic costs and carbon consumption by secondary metabolic pathways that produce these compounds. The investment of carbon in terpene synthesis can account for up to 10% (Peñuelas & Llusià, 2003) or even 20% (Sharkey & Loreto, 1993) of the carbon fixed by photosynthesis, indicating that the cost to plants can be a significant fraction of total plant production. In addition to stimulating the biosynthesis of terpenes, higher temperatures can enhance photosynthesis, which may partially compensate for the relative carbon cost of terpene production in the absence of other limiting factors, such as drought. The positive effect of enhanced signals for pollinators combined with the negative effect of higher carbon costs of enhanced floral emissions would likely lead to changes in plant fitness.

In addition, qualitative changes in floral scents such as those found here may potentially interfere with the chemical communication between plants and pollinators (Beyaert & Hilker, 2014). The effect of qualitative changes in floral scents on pollinators may depend on the learning capabilities and innate preferences of the pollinators (Cunningham *et al.*, 2004; Schiestl & Johnson, 2013). Pollinators with a high capacity to learn the floral odors of the plant species in the community may be more plastic and would adapt better to qualitative changes in floral scents, while those that rely more on innate constitutive olfactive preferences, inherited through the coevolution of their sensory systems with the floral emissions of their host plants, may be affected more (Cunningham *et al.*, 2004; Schiestl & Johnson, 2013). In effect, learning new signals could give insect pollinators the flexibility to visit species for which they do not have an innate attraction, and this capability could allow them to exploit a dynamic floral environment (Riffell *et al.*, 2013). Moreover, olfactive learning could help pollinators to adapt to subtle differences in floral scents that occur within species, such as those caused by changing environmental conditions, and therefore to continue to identify the scents by their changed blends of volatiles. For pollinators that rely on olfactive learning, such as generalist social bees (Dötterl & Vereecken, 2010), changes in the ratios of floral emissions may thus not involve serious problems of identification, because these pollinators continuously learn the scents of the flowering species in the community and establish associations between their scents and the resources they offer. In contrast, specialist pollinators such as hawkmoths that visit nocturnally blooming flowers (Raguso *et al.*, 2003; Riffell *et al.*, 2008), or specialist bees visiting only one or a few host plant species (Filella *et al.*, 2011), tend to rely to various degrees on innate preferences for the species-specific floral scents of the plants they visit, and these innate preferences may have a genetic basis that is much less dynamic than the acquired knowledge obtained by learning.

Concluding remarks and future perspectives

We demonstrated that temperature also has a general positive effect on terpene emissions that is well known in leaves. The relative increases calculated for floral terpene emissions indicate that very significant increases in the amount of floral scents will likely occur in a warmer world, although species can differ greatly in the rates of increase. We observed that the relative terpene ratios also vary with temperature, thus showing that temperature increase has the potential to induce qualitative changes in floral scent. We additionally observed seasonal changes in the effect of temperature on terpene emissions within a species. In summary, the amount of floral emissions may increase, with higher temperatures leading to enhanced olfactive signals for pollinators. The relative compositions of floral scents may also

change to different degrees in different species, which could potentially interfere with the correct identification of flowers by pollinators.

The effect of temperature on foliar emissions has been extensively explored, but the effect on floral emissions has not, so further experiments to test the observed trends in other plant species are warranted. Parallel tests of pollinator responses for determining the effect of the observed changes in floral scent on the identification of flowers by pollinators are also warranted.

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Chapter 8. Ambient ozone degrades floral scent and reduces pollinator attraction to flowers

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Abstract

Ozone is a common atmospheric pollutant that is increasing due to anthropogenic activity and has many negative impacts on the environment. In this work we analyzed the degradation of floral scent volatiles from *Brassica nigra* by reaction with ozone along a distance gradient. For this purpose we used a reaction system comprising three reaction tubes where we conducted measurements of floral VOCs by PTR-TOF-MS and GC-MS. The chemical analyses revealed a general negative effect of ambient ozone concentration and distance of exposure on floral VOC concentrations. Under the highest ozone (O_3) concentration tested (120ppb) and maximal distance of exposure measured (4.5m), monoterpenes and anisaldehyde showed degradation levels of up to 25%, while phenol and p-cymene concentrations decreased by 30%. These results reveal different reactivities with ozone for different floral scent constituents, which emphasizes that ozone exposure not only degrades floral scents but also changes their relative composition by degrading some VOCs faster than others. We also tested the effects of floral scent degradation on the responses of the generalist pollinator *Bombus terrestris* by conducting a series of behavioral tests. Behavioral tests revealed that floral scent lost its attractive effect on pollinators when exposed to 120ppb O_3 over a certain distance. The combined results of chemical analyses and behavioral responses of pollinators strongly suggest that high ozone concentrations may have significant negative impacts on plant pollination by reducing the distance over which floral olfactory signals can be detected by pollinators.

Keywords: *Brassica nigra*, *Bombus terrestris*, monoterpenes, anisaldehyde, phenol, p-cymene, behavioral tests.

Introduction

Volatile organic compounds (VOCs) mediate several ecological interactions established by plants with other organisms (Dudareva *et al.*, 2006). One of these ecological interactions mediated by VOCs is the communication of entomophilous plants with their respective pollinators (Farré-Armengol *et al.*, 2013). For the establishment of such interaction plants rely on a series of signals that allow from short to long-distance chemical communication, by emitting scent cues that are identifiable by pollinators. These chemical cues can provide diverse information to pollinators, such as the species to which they belong, their rewards availability and quality (Howell & Alarcón, 2007; Wright *et al.*, 2009), flower ontogeny (Mactavish & Menary, 1997; Goodrich *et al.*, 2006) and pollination state (Negre *et al.*, 2003). Floral scent cues also serve pollinators to locate the emitter source (flower) via scent trails that constitute concentration gradients (Cardé & Willis, 2008; Riffell *et al.*, 2008).

Ozone is a powerful oxidizing agent and a common atmospheric pollutant in the lower atmosphere that may disturb these floral scents. Tropospheric ozone concentration has significantly increased since the Pre-industrial Era due to anthropogenic activity (IPCC, 2001), and it is predicted to increase more in the next decades, enhanced by global warming and changes in land cover (Val Martin *et al.*, 2014). Ozone has harmful effects on living organisms (Mcgrath *et al.*, 2001; Kampa & Castanas, 2008; Díaz-de-Quijano *et al.*, 2012). But in addition, many studies have recently reported that ozone and other common oxidative pollutants, such as hydroxyl and nitrate radicals, affect the emissions of VOCs from plants and the interactions they mediate (Pinto *et al.*, 2007, 2010; McFrederick *et al.*, 2009; Blande *et al.*, 2010, 2011; Fuentes *et al.*, 2013). Tropospheric ozone can affect plant emissions and their effectiveness by two ways: first, by affecting the plant physiology and inducing changes in the emission profiles (Andermann *et al.*, 1999; Peñuelas & Llusia, 1999; Holopainen & Gershenzon, 2010), and second, by interacting and reacting with the emitted compounds once they are released.

The oxidative degradation of the VOCs emitted by flowers may reduce their concentration in the odor plume, decreasing the distances they can travel before reaching concentrations that are undetectable for pollinators (McFrederick *et al.*, 2008). Moreover, the reactivity of each single volatile compound with ozone is different and this implies that they may be degraded at different rates in an ozone polluted environment or in a diesel fume (NO and NO_2) polluted environment (Girling *et al.*, 2013), leading to changes in the original VOC ratios of the floral scent (McFrederick *et al.*, 2009). The oxidative reactions of ozone with plant-emitted VOCs lead to the formation of new degradation organic compounds that can be volatile and therefore persist mixed in the altered volatile blend (Pinto *et al.*, 2010). These *de novo* compounds that are not part of the original scent of the species may induce confusion to the receptor of the signal, which is the pollinator, if it is able to detect its presence. All these processes caused by the reaction of ozone with VOCs may reduce the intensity of floral scent and provide significant additional variability to flower olfactive signals once they are released, negatively contributing to flower scent reliability.

The objective of this work is to analyze the effects of exposure to different ambient ozone concentrations on the floral scent of *Brassica nigra*, while testing the effects of induced changes on the attraction of the generalist pollinator *Bombus terrestris*. *Bombus terrestris* is one of the most abundant and widespread bumblebee species in the West Palearctic and develops a very relevant role as pollinator in wild and cultivated plant communities (Rasmont *et al.*, 2008). The flower foraging preferences of *B. terrestris* present a wide degree of generalism, which converts them in a good pollination vector for a wide range of entomophilous plant species (Fontaine *et al.*, 2008). We expected flower scent to suffer quantitative and qualitative changes when exposed to ozone-enriched ambient air. We hypothesized that flower scents would experience faster reduction with distance of exposure under higher ozone concentrations. We also hypothesized that floral VOC mixtures might experience qualitative changes due to variation in the relative ratios of the existent compounds that present different reactivities with ozone, and also due to additions of new compounds resulting from oxidative reactions of VOCs with ozone. With respect to flower-pollinator communication, we hypothesized that pollinators would be more attracted to floral scent when it had not been exposed to ozone, than after being exposed to ozone-enriched ambient air for a considerable distance. We also hypothesized that under ozone-enriched ambient air, pollinators might recognize the floral scent better at shorter distances.

Materials and Methods

Brassica nigra plants and flower collection

The experiments were conducted from June to July 2014 at the University of Eastern Finland's Kuopio Campus. *Brassica nigra* plants were grown from seed harvested from wild populations at sites near Wageningen University, the Netherlands. Plants were grown individually in 1L plastic pots filled with a 3:1 mix of peat and sand and grown under greenhouse conditions with an approximate regime of light/dark cycle: 18h/6h, day temperature 23°C and night temperature 18°C and relative humidity 60%-80%. The plants were watered daily and fertilized with 0.1% 5-Superex (N:P:K 19:5:20) (Kekkilä, Finland) twice per week. Seeds were sown weekly to yield a constant supply of flowering plants (20 per week) throughout the experimental period. On each sampling day a bunch of inflorescences were cut at the greenhouse, put into a glass with water and transported to the lab for chemical measurements and/or behavioral tests.

Chemical measurements

Experimental design

We submitted the flower VOC emissions to 3 different ozone concentrations, being 0, 80 and 120ppb O_3. For each ozone concentration tested, we conducted measurements of VOC concentrations with PTR-TOF-MS at 4 distances from the scent source within the reaction system (0m, 1.5m, 3m and 4.5m) (Figure 1). We repeated the measurements of VOC concentrations with eight different flower samples of 1–2.5gDW. We used STATISTICA 8 to conduct general linear models testing the effect of ozone concentration and distance on floral VOC concentrations.

Ozone reaction system

We used an ozone reaction system composed of three glass tubes of 1.5m length and 5.5cm of inner diameter that were connected with metal tubes of 4mm inner diameter. The system let us collect the air from 4 different distances from the emission source (Figure 1). We used a filter system of activated carbon to obtain air completely free of VOCs. The cut flowers were put into a glass jar where an incoming flow of 900mL/min of clean air was pumped with a mass flow controller (Alicat Scientific, AZ, USA). The clean air collected the flower emissions inside the jar and was directed to the reaction system. Before reaching the first reaction chamber, a tube connected to an ozone generator (Stable Ozone Generator, SOG-2; UVP, LLC-Upland, CA, USA) with a flow rate of 50mL/min regulated by a mass flow controller joined the tube with the flower emissions. After ozone addition, there was the first port from which air samples could be taken for chemical measurements and behavioral tests. The first port was named "distance 0", where flower scent still had not reacted with ozone. After this point, the reaction system continued with the three reaction chambers, one after another, with one port after each chamber (distances 1, 2 and 3, at 1.5m, 3m and 4.5m respectively) and an outlet at the end connected to an ozone scrubber. We used Teflon tubes of 4mm inner diameter to connect the pump, the VOC filter, the ozone generator and the flower jar to the reaction system. We used an Ozone analyzer (Dasibi 1008-RS; Dasibi Environmental Corp., Glendale, CA, USA) to calibrate and check the ozone concentrations tested inside the reaction system.

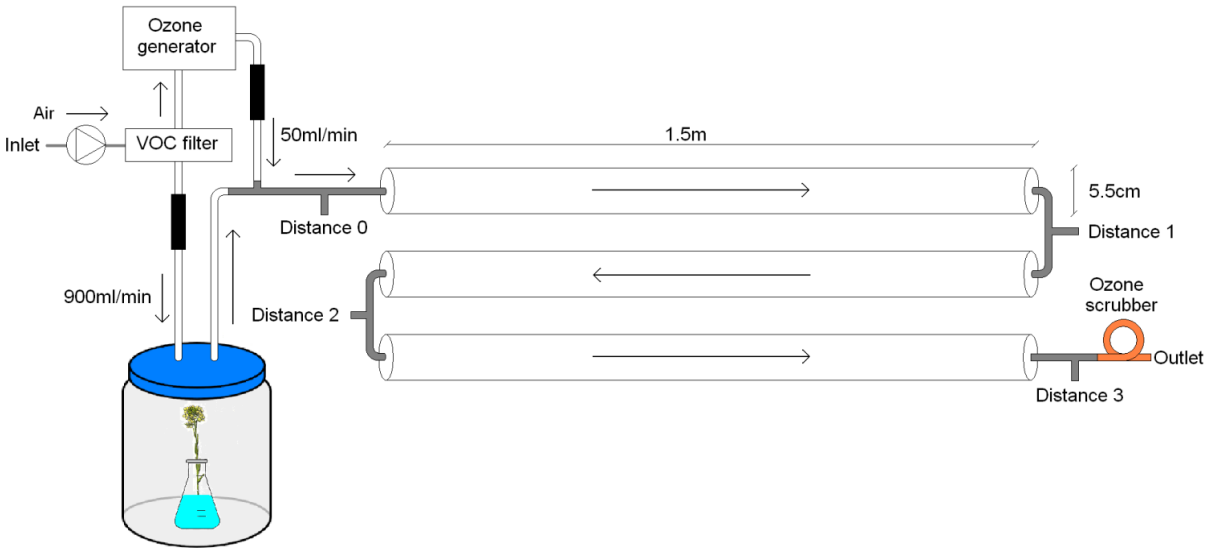

Figure 1. Schematic of the ozone reaction system. Arrows indicate the direction of the air flow. A circled triangle represents the pump. Black boxes represent mass flow controllers.

PTR-TOF-MS measurements

A high-resolution proton-transfer reaction time-of-flight mass spectrometer (PTR-TOF 8000, Ionicon Analytik, Innsbruck, Austria) was used to monitor floral VOC concentrations. Sample air from the chamber was introduced into the PTR drift tube via a 1.5 m length (outside diameter 1/16 inch) of heated (60°C) PEEK tubing at a flow rate of 200 ml min^{-1}. The PTR-TOF mass spectrometer was operated under controlled conditions (2.3 mbar drift tube pressure, 600 V drift tube voltage and 60°C temperature). The raw PTR-TOF data were post-processed with the PTR-MS Viewer 3.0.0.99 program (Ionicon Analytik). Concentrations were calculated by the program using a standard reaction rate constant of 2×10^{-9} cm^3 s^{-1} $molecule^{-1}$.

GC-MS measurements

We sampled adsorbent tubes for GC-MS analyses of floral terpene emissions. Adsorbent tubes were filled with Tenax® and Carbopack™ (150mg each; Markes International, Llantrisant, RCT, UK). We used a sampling air flow of 200mL/min and sampling times of 30−40min. The VOC samples were analysed by GC-MS (Agilent 7890A GC and 5975C VL MSD; New York, USA). Trapped compounds were desorbed with an automated thermal desorber (TD-100; Markes International Ltd, Llantrisant, UK) at 250℃ for 10 min, cryofocused at -10℃ and then transferred in a splitless mode to an HP-5 capillary column (50 m × 0.2 mm; film thickness 0.33 µm). Helium was used as a carrier gas. Oven temperature was held at 40℃ for 1 min, then programmed at 5℃ min^{-1} to 210℃, and then at 20℃ min^{-1} to 250℃ under a column flow of 1.2 ml/min. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 35-350 m/z with a scan rate of 5.38 scan/s.

Testing the response of pollinators

Experimental design

We conducted behavioral tests to test the preference between the three following pairs of air samples: "*floral scent from distance 0 at 0ppb O_3*" vs. "*clean air*", "*floral scent from distance 3 at 120ppb O_3*" vs. "*clean air*", and "*floral scent from distance 0 at 120ppb O_3*" vs. "*floral scent from distance 3 at 120ppb O_3*". For the tests from the two first comparisons, which tested floral scent against clean air, we used by one side the floral scent after traveling the corresponding distance inside the reaction system, and on the other side we took clean air that was first filtered and then passed through a glass jar with a pot of water. We conducted χ^2 tests to analyze the existence of pollinator preferences between compared air samples. We used paired t-tests to compare pollinator visitation between the artificial flowers of compared air samples.

Bombus terrestris

For the behavioral tests we used the bumblebee, *B. terrestris*, which was obtained as a group of three colonies each with a queen and providing an estimated 350-400 individuals, including adult workers, pupae, larvae and eggs (TRIPOL, Koppert Biological Systems, Netherlands). The bumblebees were kept in two conjoined ventilated polycarbonate cages giving a total foraging area of 1.4 m x 1m x 0.7m. The box containing the bumblebee colonies was put in one cage and the other cage was used to provide *B. nigra* flowers and a 50% sucrose solution to feed the bumblebees. We regularly provided fresh *B. nigra* flowers to the bumblebees to familiarize

them with the floral scent and associated reward. The colonies remained in healthy condition and provided adult individuals that were suitable for behavioral tests throughout the 1 month period of the behavioral study.

Behavioral chamber

Behavioral tests were conducted in a cylindrical chamber made of transparent polycarbonate with a 1m height and 1.5m diameter (Figure 2). The lateral walls of the chamber were covered with light green paper to avoid interferences in bumblebee behavior due to visual interferences from the outside of the chamber. Two lamps were put on the top of the behavioral chamber on opposite sites and were used as a light source. The chamber had a 20cm x 30cm window. Two metal tubes of approximately 1m length and 4mm of inner diameter were inserted into the cage coming from the top and placed at opposite edges of the chamber. The metal tubes were connected to the two incoming air sources that we wanted to test against each other inside the behavioral chamber. Metal tubes had some holes on the lower part to release the air close to the artificial inflorescences we placed on the ground of the chamber. We made artificial inflorescences that resembled those of *B. nigra*. We cut yellow non-scented paper with the shape of petals and attached it to a white thin Tefflon tube that worked as a stem using needles. Each inflorescence was composed of 8 flowers with an alternate disposition. The inflorescences were put on the ground close to the metal tubes that released the air sources tested, standing on a metal support. A third metal tube of the same dimensions was inserted in the center of the chamber. This tube had many holes all along its length oriented to all directions and was connected to a pump to make the air come out from the chamber (Figure 2).

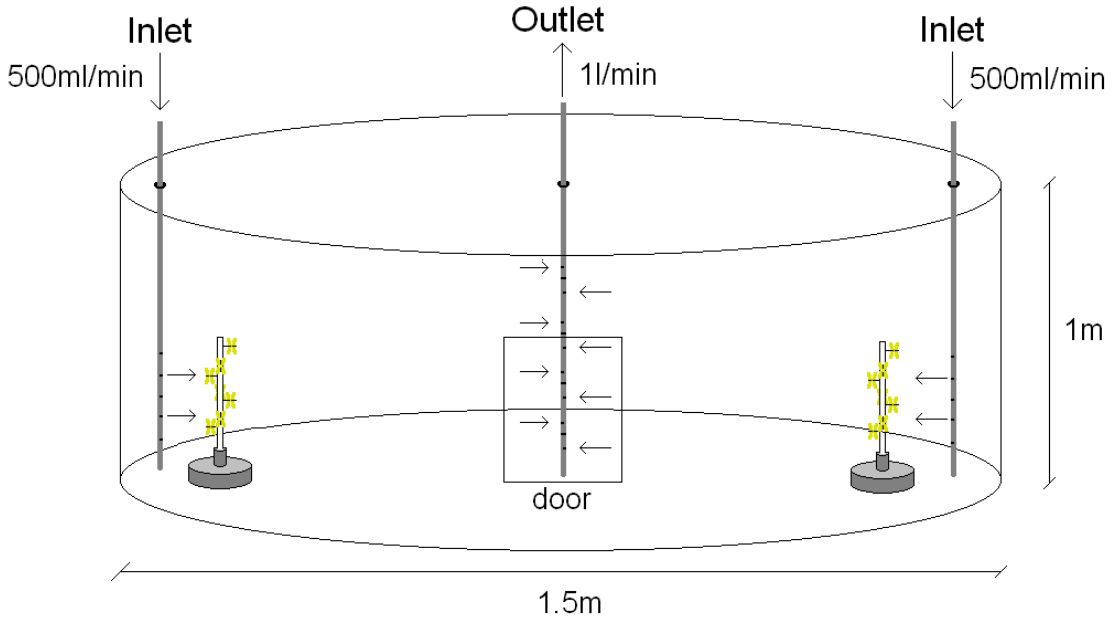

Figure 2. Behavioral test chamber. Arrows indicate the direction of air flow.

Behavioral tests

Before behavioral tests started we conducted a series of checks and calibrations. First, we prepared and turned on the reaction system and we waited for it to reach a steady state, by measuring the floral VOC concentrations with the PTR-TOF-MS and checking when they

stabilized. After that we connected the two air sources that we wanted to test to the behavioral chamber. The pumps were turned on and the two incoming air flows were adjusted to 500mL/min and the outlet central tube to 1L/min (Figure 2). We then waited for another 30 minute period for the stabilization and homogenization of the air flows and VOC concentrations in the behavioral chamber system. After that the behavioral tests started. Each time one bumblebee was collected from the colony and put in a small pot. It was immediately transported to the lab were the behavioral chamber was placed, always in dark conditions, and it was released in the middle of the chamber through the window. Once the test started the two lamps were turned on and we waited a few seconds for the bumblebee to fly, at which point the clock was started and the bioassay was continued for 10 minutes. The chamber was divided into two halves – each containing an odour source and an artificial inflorescence fashioned from a length of Teflon tubing, yellow paper and brass pins – and the time spent in each half was recorded. We also recorded the number of visits that the bees made to the artificial inflorescences. A visit was considered to have occurred when a flying bumblebee landed on one of the artificial inflorescences. Short flight movements between flowers within the same inflorescence were not considered to be different visits. If the bumblebees left the inflorescence, flew in the open chamber and landed again, we considered it a new visit. In addition, we transformed the data on pollinator visitation into a binary variable (0/1) for the statistical analyses. We assigned the value zero when no visits were conducted to artificial flowers during the test and we assigned the value one when pollinators conducted one or more visits. Once the test finished we released the bumblebees in a separate cage to avoid using the same individual for different test replicates on the same day, and we took a new bumblebee for the next trial.

Results

Effects of ambient ozone on the chemistry of floral emissions

Ozone concentration and distance of exposure had a negative effect on the concentration of flower scent volatiles (Figure 3). Monoterpene (m/z 137.133), anisaldehyde (m/z 137.1562), and phenol (m/z 95.1194) concentrations showed very significant negative correlations with ozone concentration (P<0.0001), distance (P<0.0001) and the interaction between ozone concentration and distance (P<0.0001). Also, p-cymene (C10H14, m/z 135.1174) concentration showed a very significant negative correlation with ozone concentration (P<0.0001) and distance (P=0.013). Oppositely, benzaldehyde (m/z 107.0497) concentration increased with ozone concentration and distance, although the effects were not found to be significant (Figure 4).

Under the highest ozone concentration tested, at the longest distance from the scent source (4.5m), monoterpene concentration decreased 26.4%, anisaldehyde decreased 27%, phenol decreased 29.5%, p-cymene decreased 31% and benzaldehyde increased 17%. These compound-specific responses lead to changes in floral VOC relative composition. Floral terpene composition showed gradual changes with distance when exposed to ozone, although changes were not found to be significant (Figure 5). The monoterpenes β-myrcene, β-thujene, (Z)-β-ocimene and γ-terpinene showed gradual relative increases with respect to other terprene compounds when exposed to increasing ozone concentrations, while 1R-α-pinene gradually decreased.

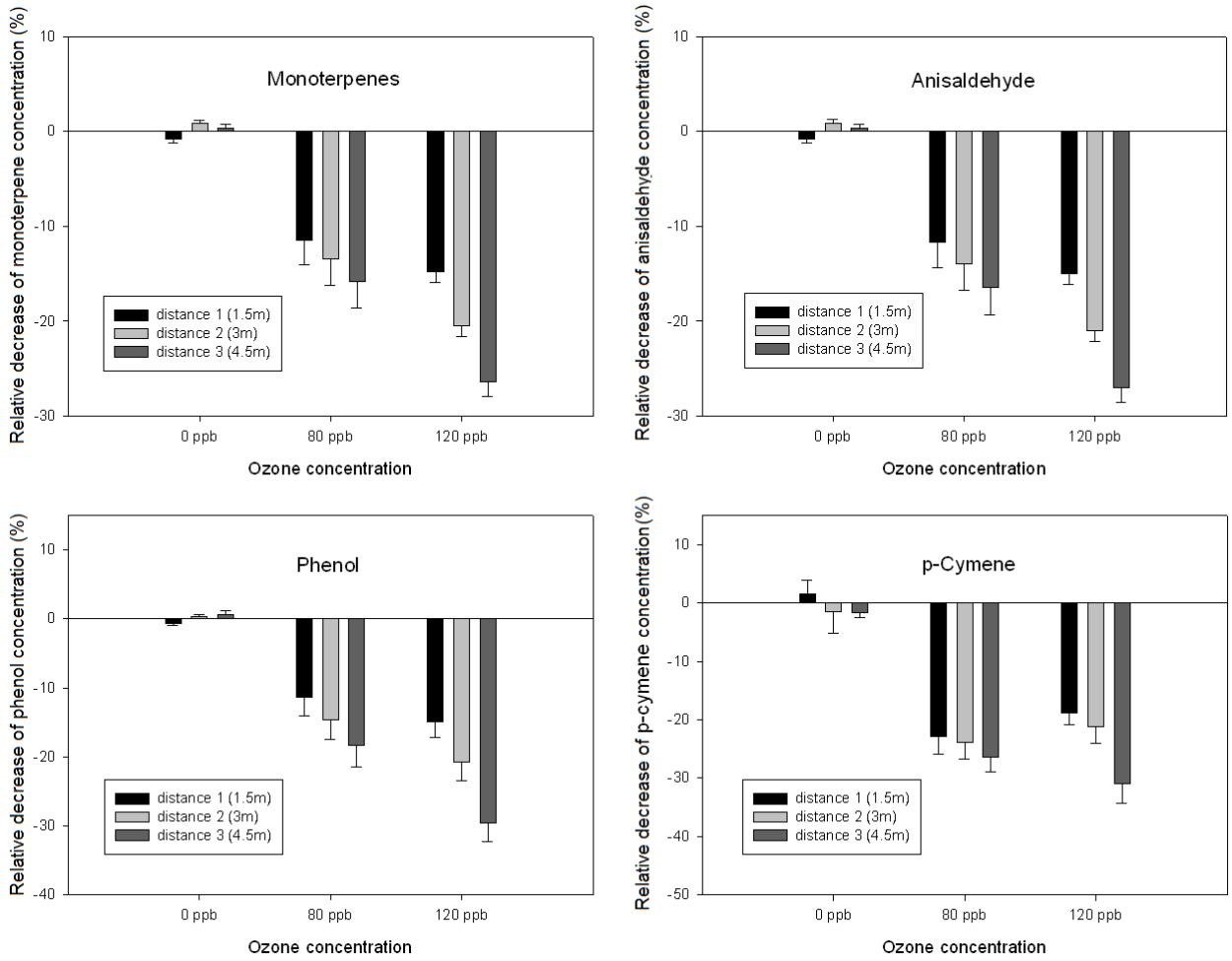

Figure 3. Floral scent degradation in response to the exposure to ozone. The figure shows the relative decrease in monoterpene, anisaldehyde, phenol and p-cymene concentrations of *Brassica nigra* floral scent exposed to different ozone ambient air concentrations (0ppb O_3, 80ppb O_3, 120ppb O_3) at different distances from the emitter flower source (1.5m, 3m, 4.5m). Error bars indicate SEM.

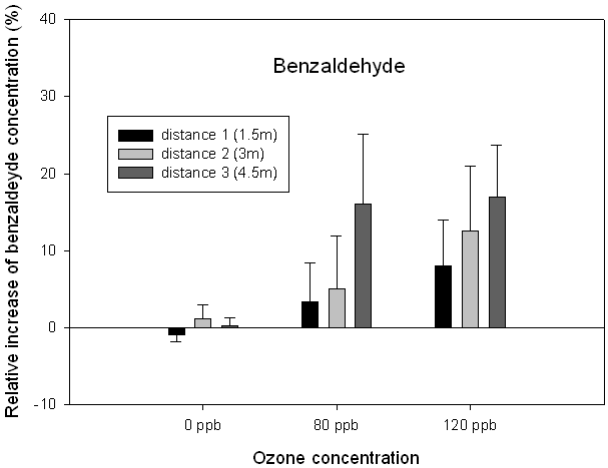

Figure 4. Relative increase in benzaldehyde concentrations of *Brassica nigra* floral scent exposed to different ozone ambient air concentrations (0ppb O_3, 80ppb O_3, 120ppb O_3) at different distances from the emitter flower source (1.5m, 3m, 4.5m). Error bars indicate SEM.

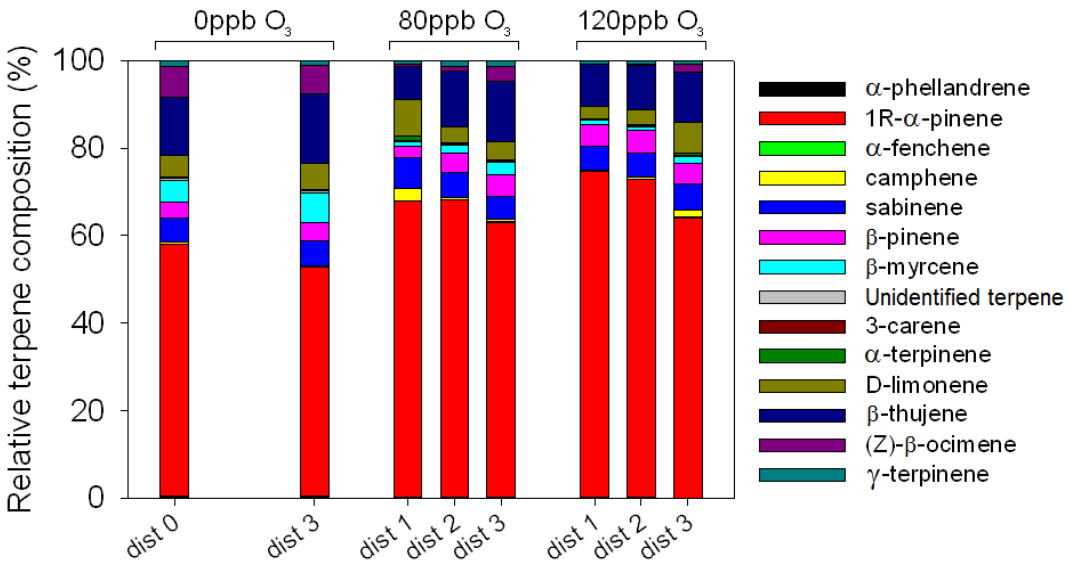

Figure 5. Relative terpene composition (%) of *Brassica nigra* floral scent at different distances from scent source under different ozone concentrations (*N*= 2, 3, 2, 2, 4, 2, 2, 4).

Pollinator responses to behavioural tests

Bumblebees showed a clear preference for "*floral scent from distance 0 at 0ppb O_3*" in front of "*clean air*" (χ^2 test, *P*=0.01) (Figure 6A). From a total of 21 tests, thirteen bumblebees spent more time in the half with "*floral scent from distance 0 at 0ppb O_3*", three of them spent more time in the half with "*clean air*", and five individuals did not make a clear choice. On the other hand, bumblebees showed no clear preferences between "*floral scent from distance 3 at 120ppb O_3*" and "*clean air*" (χ^2 test, *P*=0.37) (Figure 6B). From a total of 22 tests, eight bumblebees spent more time in the half with "*floral scent from distance 3 at 120ppb O_3*", twelve of them spent more time in the half with "*clean air*", and two individuals did not make a clear choice. Finally, bumblebees showed a marked preference for "*floral scent from distance 0 at 120ppb O_3*" in front of "*floral scent from distance 3 at 120ppb O_3*" (χ^2 test, *P*=0.005) (Figure 6C). From a total of 21 tests, fifteen bumblebees spent more time in the half with "*floral scent from distance 0 at 120ppb O_3*", three of them spent more time in the half with "*floral scent from distance 3 at 120ppb O_3*", and three individuals did not make a clear choice.

Bumblebees realized landings on artificial flowers in some of the tests conducted (Figure 7). The results show that more bumblebees conducted landings on artificial flowers associated with "*floral scent from distance 0 at 0ppb O_3*" than on artificial flowers associated with "*clean air*" (paired t-test, *P*=0.04) (Figure 7A). More bumblebees landed on artificial flowers associated with "*floral scent from distance 3 at 120ppb O_3*" than on artificial flowers associated with "*clean air*", but the difference was not significant (paired t-test, *P*=0.08) (Figure 7B). Finally, more bumblebees conducted landings on artificial flowers associated with "*floral scent from distance 0 at 120ppb O_3*" than on artificial flowers associated with "*floral scent from distance 3 at 120ppb O_3*" (paired t-test, *P*=0.01) (Figure 7C).

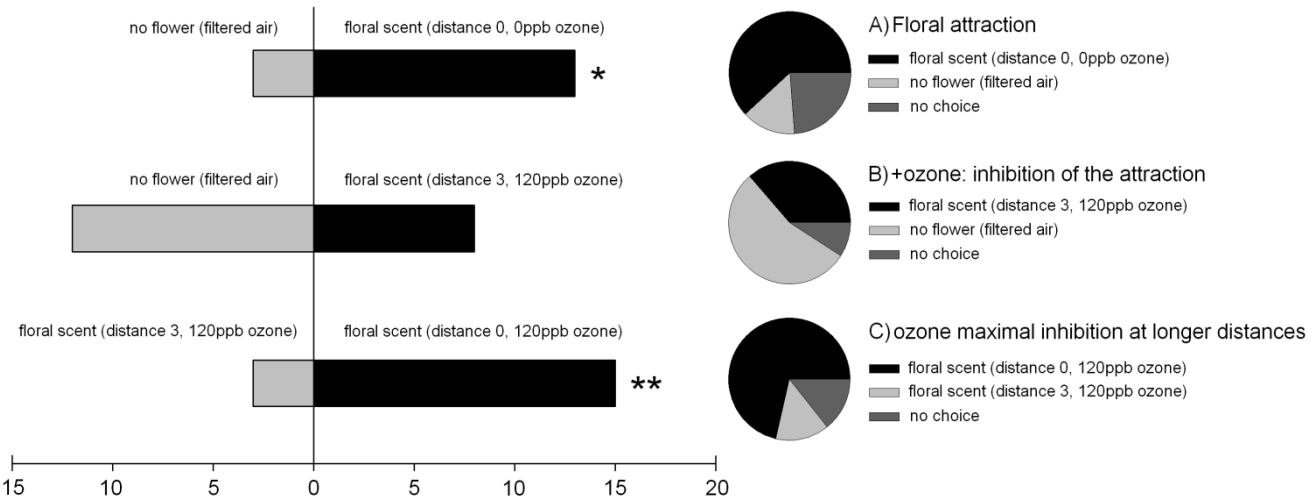

Figure 6. Pollinator olfactive preferences for the tests comparing: A) unaltered floral scent (distance 0 at 0ppb O_3) vs. clean air (filtered air with no flower scent) (*n*=21); B) degraded floral scent (distance 3 at 120ppb O_3) vs. clean air (filtered air with no flower scent) (*n*=24); C) unaltered floral scent (distance 0 at 120ppb O3) vs. degraded floral scent (distance 3 at 120ppb O_3) (*n*=21). Asterisks indicate the level of significance of χ^2 tests (*P<0.05; **P<0.005).

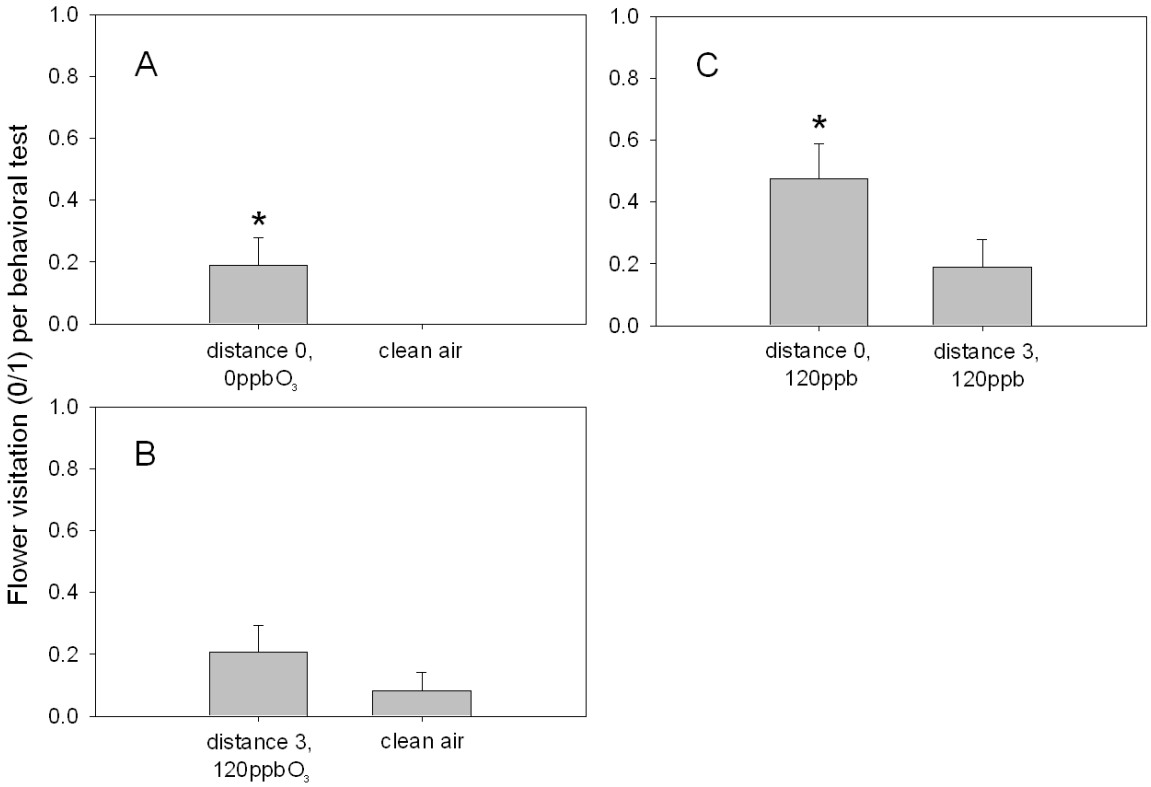

Figure 7. Pollinator visitation to artificial flowers for the behavioral tests comparing: A) unaltered floral scent (distance 0 at 0ppb O_3) vs. clean air (filtered air with no flower scent) (*n*=21); B) degraded floral scent (distance 3 at 120ppb O_3) vs. clean air (filtered air with no flower scent) (*n*=24); C) unaltered floral scent (distance 0 at 120ppb O3) vs. degraded floral scent (distance 3 at 120ppb O_3) (*n*=21). Asterisks indicate the level of significance of paired t-tests (*P<0.05). Error bars indicate SEM.

Discussion

Quantitative and qualitative changes in emitted floral scents after exposure to ozone

The concentrations of floral VOCs experienced significant reductions with distance when exposed to ozone enriched ambient air. The main floral volatiles of *B. nigra* experienced significant decreases that ranged from 25 to 30% when exposed to 120ppb O_3 for 4.5m in the reaction chamber. To our knowledge this is the first work to provide experimental evidence and quantification of floral scent degradation with ozone exposure. McFrederick et al. (2008) previously published a theoretical work modeling the degradation of three common floral monoterpenes under different concentrations of ozone and hydroxyl and nitrate radicals, whose predictions are mostly in accordance with our results.

The different floral VOCs presented variable degrees of degradation, which could be explained by their different reactivities with ozone (Atkinson *et al.*, 1995; Atkinson & Arey, 2003). The diverse reactivities with ozone shown by different floral VOCs may imply subtle changes in relative composition of floral blends when exposed to ozone-enriched ambient air for a certain distance. In fact, we detected some changes in the terpene relative composition of floral scent with ozone concentration and distance, although they were not found to be significant maybe due to low statistical power (Figure 5).

Effects of ozone-related changes in floral scent on the attraction of pollinators

Our results on the behavioral response of pollinators clearly point to a loss of attraction to floral scent cues when they have been exposed to ozone. When comparing the response of *B. terrestris* to unaltered floral scent against clean air with no scent we observed a clear preference for the first option (Figure 6A) and for the artificial flowers associated with it (Figure7A), thus confirming the existence of a clear attraction effect of floral scent on pollinators. We later compared the response of *B. terrestris* to floral scent exposed to high ozone concentration for long distance against clean air with no scent and pollinators showed no preference for any of the two options (Figures 6B, 7B). This clearly suggests that long exposition of floral scent to high ozone concentrations led to a loss of attraction effect on pollinators. Finally, we compared the response of *B. terrestris* to floral scent mixed with high ozone concentration before having time to react and after long distance of exposure, and we observed that pollinators clearly preferred the first option (Figure 6C) and visited more the artificial flowers associated with it (Figure 7C), which strongly supports that attraction to floral scent is gradually reduced with distance under high ozone ambient concentrations.

We observed a significant degradation of floral scent cues with ozone that may justify in great measure the loss of the attraction effect on pollinators. High ambient ozone concentrations like those that we tested here may cause a significant reduction of the distance that floral chemical cues can travel before reaching concentration levels that are below pollinator olfactive detection limits. This may be translated into a significant reduction in the distance from which floral chemical cues can be perceived by pollinators. This assumption is strongly supported by the finding that high tropospheric ozone concentrations can reduce the distance for plant-to-plant communication via volatiles (Blande *et al*., 2010) and also affect tritrophic interactions involving herbivore-damaged plants and carnivores (Pinto *et al*., 2007).

Qualitative changes in floral scent composition may also lead to confusion with pollinator innate or learned olfactory preferences. It is important for insect-pollinated plants to maintain a good level of reliability in their floral signals directed to pollinators, throughout the

maintenance of low levels of variability. Such low levels of variability in floral traits of flowers have been postulated to be beneficial for reward-offering plants (Salzmann *et al.*, 2007). Pollinators promote stabilizing selection on floral traits of rewarding flowers, due to the advantages that flower constancy bring to both pollinators (higher foraging efficiency) and plants (less deposition of heterospecific pollen in the stigmas) (Gegear & Laverty, 2005).

Implications of floral scent degradation by increasing tropospheric ozone concentrations

The increase in tropospheric ozone since the Pre-industrial Era is estimated to be around 35% with subtle differences among regions (IPCC, 2001). Mean annual tropospheric ozone concentrations over the mid latitudes of the Northern Hemisphere currently range between 20 and 45ppb (Vingarzan, 2004). However, ozone concentrations are significantly higher in some urban areas (Kleinman *et al.*, 2002), where they can reach or surpass 120ppb, the highest ozone concentration that we tested in our experiments. Thus, the effects revealed by our work may be especially relevant for those regions with high tropospheric ozone concentrations. Among the plant communities experiencing the most relevant effects we may find agricultural lands close to urban areas where pollination efficiency can be limited. The most important concerns rising from these results may include reduced crop productivity and the disruption of several ecological processes related with pollination in plant communities affected by ozone pollution.

Conclusions

Our results strongly suggest that ozone can have significant negative effects on pollinator attraction to flowers. High ozone ambient air concentrations caused fast degradation of *B. nigra* floral scent with distance, reducing the distance range from which they can be detected by pollinators. Moreover, ozone exposure induced qualitative changes in floral scent composition, which can cause confusion in the olfactive recognition of the signal by pollinators. Behavioral tests conducted with *B. terrestris*, a common and widespread generalist pollinator, confirmed that ozone concentrations of 120ppb, which can frequently occur near big urban areas, can strongly inhibit pollinator attraction to flowers.

Aknowlegements

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General conclusions

1. Floral olfactive cues act in concert with visual cues and tactile and gustatory stimuli to attract pollinators. Apart from pollinator attraction, floral volatiles play multiple relevant roles on the ecological interacions of flowers with different organisms. Floral VOC emissions show spatial and temporal patterns of change that reflect specific functions and constitute a big source of variability. Furthermore, floral VOC emissions can be affected by many environmental factors. Floral VOC emissions are predicted to increase in response to most of the drivers of Global Change.

2. The floral emissions of entomophilous plants are quantitatively and qualitatively variable, with species emiting strong and complex floral scents, but also others with weak and very simple scents, while anemophilous species tend to present low floral emission rates and lower VOC richnesses than entomophilous plants. This leads to the conclusion that biotic pollination is a major factor selecting for the appearance of strong and complex floral VOC emissions.

3. Seasonal patterns of decrease in plant competition for pollinator attraction can lead to phenological patterns of decrease in the amounts of floral rewards offered by co-occurring plants in the community, especially in species with long flowering periods. Floral volatile emissions that indicate the presence of flowers and the associated rewards did not follow the same decreasing pattern at the species level.

4. Optimum temperatures for floral terpene emissions are adapted to the temperature conditions during the flowering period of each species. Species flowering in winter present lower optimum temperatures than species flowering in warmer seasons.

5. Floral microbiota can play a relevant role in the emission of floral scent, as demonstrated in *Sambucus nigra* flowers, where the removal of microorganisms caused a significant decrease in the amount of emitted floral VOCs, without affecting floral physiology and VOC contents.

6. Florivory caused strong responses on *Diplotaxis erucoides* floral emissions, with immediate increases in the rates of emission of few defensive volatiles. Folivory alone did not cause any significant change in floral emissions, but when combined with florivory the response was the highest, revealing a synergistic effect that maximizes the defensive response when the herbivore attack is generalized to the whole plant.

7. Floral volatile emissions are enhanced by temperature and are predicted to increase with Global Warming. Floral scent relative composition may change with temperature as different compounds have different responses to temperature.

8. Tropospheric ozone degrades floral volatile emissions and reduces the distance from which floral scents can effectively attract foraging pollinators to flowers.

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