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

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1 Abstract

2 Emissions of volatiles from leaves exhibit temperature dependence with maximums, but optimum temperatures for the release of floral volatiles or the mechanism of 3 optimization of these emissions have not been determined. We hypothesized that 4 flowers have an optimum temperature for the emission of volatiles and, because the 5 6 period of flowering varies highly among species, that this optimum is adapted to the 7 temperatures prevailing during flowering. To test these hypotheses, we characterized the 8 temperature responses of floral terpene emissions of diverse widespread Mediterranean plant species flowering in different seasons by using dynamic headspace sampling and 9 10 analysis with gas chromatography mass spectrometry. The floral emissions of terpenes 11 across species exhibited maximums at the temperatures corresponding to the season of 12 flowering, with the lowest optimal temperatures observed in winter-flowering and the highest in summer-flowering species. These trends were valid for emissions of both 13 14 total terpenes and the various terpene compounds. The results show that the optimum temperature of floral volatile emissions scales with temperature at flowering and 15 16 suggest that this scaling is the outcome of physiological adaptations of the biosynthetic 17 and/or emission mechanisms of flowers.

18 Keywords: flower scent, interspecific variation, phenology, seasonal variability.19

20 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

25 emission rates and composition, thereby interfering with or affecting chemical 26 communication between plants and pollinators (Farré-Armengol et al. 2013; Farré-Armengol et al. 2014). Several environmental factors can affect the emission of VOCs 27 from various plant tissues; the effects of temperature and light on foliar terpene 28 emissions are the best studied (Peñuelas and Llusià 2001; Niinemets et al. 2004; Grote 29 30 et al. 2013). The responses of terpene emissions from leaves to temperature are well 31 characterized (Niinemets et al. 2010) and are known to be determined by temperature dependencies of the physicochemical properties of terpenes, such as volatility, solubility 32 and diffusivity, and by the effects of temperature on foliar physiology, such as terpene 33 34 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 35 known. However, we argue here that the need of maximization of the intensity of floral 36 37 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 38 39 temperature ranges to which the flowers of each species are typically exposed.

40 Species from cooler environments have lower optimum temperatures for 41 photosynthesis than do species living in warmer environments, which reveals a positive 42 correlation between species-specific optimum temperature for photosynthesis and the range of ambient temperatures in which the species live (Berry and Björkman 1980; 43 44 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 45 46 which individuals grow, indicating an additional physiological process of acclimation (Cleveland et al. 1992; Kattge and Knorr 2007). In species that do not store terpenes, 47 48 the rates of terpene emission have temperature response curves similar to those of 49 photosynthesis (Copolovici and Niinemets 2005; Llusia et al. 2006; Niinemets et al.

2010). In fact, terpene biosynthesis and physiological processes related to the emission 50 51 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 52 53 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; 54 55 Niinemets 2004). We hypothesized that plant species may thus be expected to 56 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 57 flowering. In this study, we aimed to test this hypothesis in Mediterranean species 58 59 flowering at different times of the year.

Most Mediterranean angiosperms flower in spring. Some species, however, 60 flower in summer, autumn or even winter. Flowers are thus exposed to different 61 temperature ranges and can potentially evolve different temperature sensitivities of their 62 63 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 64 temperature ranges. In contrast, summer-flowering species may adapt their floral 65 66 emissions to high temperatures. Such different responses can result from differences in the composition of volatiles emitted by the species and from physiological 67 modifications in the production and release of volatiles. 68

We tested the 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 for which we had previously studied the responses of floral BVOCs emission rates to warming (Farré-Armengol et al. 2014). 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.

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79 Methods

80 Study site and species sampled

81 The study was conducted at various field locations within the province of Barcelona

82 (Catalonia, Spain). Six common Mediterranean species of anemophilous plants in

63 Garraf national park (UTM: 31T, 409km, 4570km; *Dorycnium pentaphyllum* Scop.,

84 Erica multiflora L., Globularia alypum L.) and Cerdanyola del Vallès (UTM: 31T,

426km, 4595km; Spartium junceum L., Sonchus tenerrimus L., Dittrichia viscosa (L.)

Greuter), and one anemophilous plant in Collserola national park (UTM: 31T, 427km,

4592km; *Quercus ilex* L.) were included in the analysis. Floral emissions from *D*.

viscosa were collected in late summer and again in early autumn. In each of the two

series of measurements conducted on *D. viscosa* we sampled individuals from two

90 different populations from very close locations (2-3 km) in Cerdanyola del Vallès. The

species sampled include a wide range of flowering periods with different mean

92 temperatures (Table S1, Suppl. Mat.). For a same location, we measured floral

93 emissions for species flowering during different seasons.

94

95 *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.

104 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. 105 pentaphyllum: 10-15 flowers, S. junceum: 4-5 flowers, S. tenerrimus: 1 capitula, D. 106 107 viscosa: 5-9 capitula). We used two different chambers depending on the size of the flowers of each species. A 12 cm³ chamber was used at a flow rate of 450-500 ml min⁻¹ 108 for G. alypum, E. multiflora, Q. ilex, D. pentaphyllum and S. tenerrimus, and a 175 cm³ 109 chamber was used at a flow rate of 250-300 ml min⁻¹ for *S. junceum* and *D. viscosa*. We 110 111 collected the samples of terpene emissions after setting the required quantum flux 112 density and temperature and after an acclimation period of approximately 10 min or the 113 time needed to reach a steady-state exchange of CO₂ and H₂O. The enclosed flowers were sequentially submitted to different temperatures, and their emissions were sampled 114 for additional 10 min. The air exiting the chamber of the IRGA, at a mean flux of air of 115 approximately 200-250 ml min⁻¹, was directed through a Teflon tube to a stainless 116 steel tube (89 mm in length and 6.4 mm external diameter) filled with the adsorbents 117 Tenax (114.6 mg, 50% vol.) and Carbotrap (236.8 mg, 50% vol.), separated by sorbent-118 119 retaining springs (Markes International Inc. Wilmington, USA) fixed using gauze-120 retaining springs (Markes International Inc. Wilmington, USA) and closed with air-tight 121 caps (Markes International Inc. Wilmington, USA), which collected the terpenes 122 emitted by the flower(s) over a period of 10-15 min. The same process was repeated

123	with empty chambers of the IRGA that served as blanks of the system. At least two
124	blank samples were collected for each curve, one at the beginning of the emission
125	samplings and another at the end. We collected 3-5 replicate samples of emissions per
126	species (G. alypum: 5, E. multiflora: 4, Q. ilex: 4, D. pentaphyllum: 5, S. junceum: 5, S.
127	tenerrimus: 4, D. viscosa late summer: 3, D. vicosa early autumn: 3). Each replicate was
128	collected from a different plant. At the end of each sampling sequence we collected the
129	flower samples from which emissions were collected and we dried and weighed the
130	flowers for emission rate calculations. Sampled tubes were stored in a freezer at -25°C
131	until we conducted the analyses by GC-MS.

132

133 Terpene analyses

134 The terpene samples in the adsorbent tubes were thermally desorbed using an injector 135 (Unity, Series 2, Markes International Inc. Wilmington, USA) and released with an automatic sample processor (TD Autosampler, Series 2 Ultra, Markes International Inc. 136 Wilmington, USA) to be analyzed by an Agilent gas cromatography mass spectrometry 137 138 (GC-MS) system (Agilent Technologies, GC: 7890A, MS: 5975C inert MSD with Triple-Axis Detector, Palo Alto, CA, USA). The desorbed sample was retained in a 139 cryo-trap at -25°C. The split was 2:1. The sample was desorbed again at 320°C for 15 140 and 10 min and injected into the column with a transfer line at 250°C. Samples were 141 injected into a 30 m x 0.25 mm x 0.25 µm capillary column (HP-5MS, Agilent 142 Technologies). Helium flow was 1 ml min⁻¹, and total run time was 26 min. After 143 injection, the sample was maintained at 35 °C for 1 min, the temperature was then 144

increased at 15 °C min⁻¹ to 150 °C and maintained for 5 min, then increased at 50 °C

146 min⁻¹ to 250 °C and maintained for 5 min and then increased at 30 °C min⁻¹ to 280 °C 147 and maintained for 5 min.

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149	The terpenes were identified by comparing the retention times with standards
150	from Fluka (Buchs, Switzerland) that had been injected into clean adsorbent tubes, and
151	the fractionation mass spectra were compared with standard spectra and spectra in the
152	Nist05a and wiley7n mass spectral libraries. Terpene concentrations were determined
153	from the calibration curves. Calibration curves for the common terpenes α -pinene, β -
154	pinene, limonene, γ -terpinene, linalool and α -humulene were determined daily. The
155	terpene calibration curves ($n=4$ different terpene concentrations from 0.33 10^{-4} to 0.33
156	mL L^{-1}) were always highly significant (R^2 >0.99 for the relationship between the signal
157	and the amount of compound injected).

158

159 Statistical analysis

160 We used the *loess* function of the *stats* package from R (R Development Core Team 2011) to characterize the shape of the curve of the temperature responses of floral 161 terpene emissions and to determine the optimum temperature for floral terpene 162 163 emissions. Optimum temperature for floral terpene emissions was considered to be the 164 temperature at which flowers emit the maximum terpene emission rates. The loess 165 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 166 167 data and to determine the relationship between optimum temperature for floral terpene 168 emissions (the temperature at which floral terpene emissions of a particular species

reached highest emission rates) and mean temperature of the month of the floweringpeak by linear regression models.

171

172 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.

180

181 <u>Results</u>

182 G. alypum and E. multiflora flowers emitted detectable amounts of α -pinene, camphene,

183 3-carene and D-limonene (Table S2, Suppl. Mat.). Q. ilex male flowers emitted α -

184 pinene, β -pinene, camphene, 3-carene and D-limonene. *D. pentaphyllum* flowers

emitted 3-carene, (E)- β -ocimene and (Z)- β -ocimene. S. junceum flowers emitted α -

186 pinene and α -farnesene. *S. tenerrimus* flowers emitted α -pinene and 3-carene. *D.*

187 *viscosa* flowers of late summer emitted α -pinene, β -pinene, α -phellandrene, β -

188 phellandrene, camphene, 3-carene, D-limonene, eucalyptol, γ -terpinene, α -terpinolene

- and α -thujene. *D. viscosa* flowers of early autumn emitted α -pinene, β -pinene, α -
- 190 phellandrene, camphene, 3-carene and D-limonene (Table S2, Suppl. Mat.).

191 The rates of terpene emission initially increased with temperature in all species 192 and generally reached a maximum (Fig. 1). The temperature-response curves of floral 193 terpene emissions showed species-specific differences. The rates of floral emission of 194 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 195 not decline within the range of temperatures included in our measurements. The winter-196 197 flowering species G. alypum and E. multiflora exhibited maximum floral terpene 198 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 199 200 floral terpene emission increased with increasing temperature up to 35 $^{\circ}$ C, and a 201 moderate reduction was observed at 40 °C. The rates of terpene emission in the flowers 202 of S. junceum, D. viscosa and S. tenerrimus sampled in late spring and summer 203 increased with increasing temperature, even up to 40 °C, whereas the summer flowers 204 of D. viscosa and S. tenerrimus experienced a maximum increase only from 35 to 40 °C. 205 In early autumn, the maximum emission from D. viscosa flowers was at 25-30 °C (Fig. 206 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 (Pearson's r=0.91, *P*=0.002, Fig. 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, r=0.85, *P*=0.02; camphene, r=0.91, *P*=0.03; r=0.96, β -pinene, *P*=0.17; 3-carene, r=0.88, *P*=0.008; D-limonene, r=0.99, *P*<0.001; Fig. 3).

214

215 Discussion

216 Our data demonstrate that the well-known temperature-dependent increase of terpene emissions previously reported for leaves also occurs in flowers (Fig. 1). The 217 temperature responses of floral volatile emission generally exhibited an optimum, 218 suggesting that these emissions reflect de novo synthesis of terpenes (Niinemets et al. 219 220 2010; Li and Sharkey 2013; Monson 2013). The temperature dependence function for 221 de-novo synthesized isoprenoids considers an Arrhenius type response which describes 222 a curve with an optimum (Niinemets et al. 2010). This optimum represents a threshold 223 temperature from which physiological processes involved in isoprenoid biosynthesis are 224 limited or completely inhibited. On the other hand, the emission rates for species that 225 store monoterpenes in specialized plant tissues are suggested to be controlled only by 226 physical evaporation and diffusion, two processes that do not decline but present a 227 sustained increase with temperature.

As we hypothesized, species flowering in different seasons had optimum 228 229 temperatures for floral emissions that paralleled the mean temperature of the month of the flowering peak (Fig. 2). The positive correlation between the temperature optimum 230 231 for floral emission and ambient temperature generally resembled the correlation between optimum temperature for photosynthesis and ambient temperature (Berry and 232 233 Björkman 1980; Niinemets et al. 1999; Kattge and Knorr 2007). Species flowering in 234 cold seasons had maximum emissions at lower temperatures than did species flowering 235 in warm seasons. Our results thus supported the hypothesis that the temperature 236 responses of floral terpene emissions were adapted to the temperature ranges to which 237 the flowers were exposed during flowering. Even though we were not able to determine 238 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 239

240 species, the difference between optimums for species flowering in cold and warm 241 seasons would increase, strengthening the significance of our conclusions. The faster 242 increases in floral terpene emission rates with temperature in early-flowering 243 entomophilous species show that these species are more sensitive to temperature increases than species flowering in spring or summer, which is in accordance with the 244 245 observed higher responsiveness of early-flowering plants to climate warming by 246 advancing more their flowering phenology (Dunne et al. 2003; Cleland et al. 2007). 247 Also, different flowering seasons combine changes in temperatures with changes in the length of the day (hours of daylight), which may also play a role on floral terpene 248 249 emissions (Colquhoun et al. 2013).

250 Our results also showed that the emission rates of each terpene compound also 251 tended to have an emission optimum, and that this optimum was positively correlated 252 with the mean temperature of the month of the flowering peak of that species (Fig. 3). 253 This response of the individual terpene compounds indicated that the differences in the 254 optimum temperature for total terpene emissions among species was not due to the 255 differences in the compounds that constitute the scents of flowers, but reflected 256 physiological adaptation of underlying biochemical processes. Terpene production in 257 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, 258 259 terpene production in winter-flowering species has been adapted to maximize floral 260 emissions at low temperatures. This pattern is clearly supported in the insect-pollinated 261 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 262 263 temperature for floral terpene emissions to ambient temperature of the flowering season 264 might not be exclusively linked to biotic pollination.

We observed different temperature responses of floral terpene emissions in D. 265 266 viscosa in late summer and early autumn. Dittrichia viscosa plants can flower abundantly over a long period of 4-5 weeks, which allowed us to conduct a second 267 268 series of measurements some weeks after the first measurements. The two series of measurements were thus conducted during the same flowering event, but at different 269 270 moments (Table 1, 17-25 September and 23-30 October). Analogous intraspecific 271 seasonal differences in the responses of terpene emissions to environmental conditions 272 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 273 274 individuals of the same species, at least in those species that can flower under different 275 temperature conditions, and indicate some degree of phenotypic, epigenetic or 276 genotypic plasticity in the physiology of the flowers of these species, which clearly 277 constitutes an important adaptive modification to optimize flower emissions at diverse 278 temperature ranges.

279 Such plasticity in the physiology of flowers controlling terpene floral emissions 280 could be adaptations of the terpene biosynthetic and/or release mechanisms of floral volatiles. The biosynthetic pathways involved in the production of some terpene 281 volatiles are well described (Dewick 2002; Dubey et al. 2003; Kuzuyama and Seto 282 283 2003), and the mechanisms that regulate terpene biosynthetic rates have been 284 extensively investigated (Dudareva and Pichersky 2000; Fischbach et al. 2002; 285 Dudareva et al. 2004; van Schie et al. 2006). The key controls operating in terpene 286 production are the transcription, production and activity of enzymes and the 287 concentrations of the substrates of these enzymes (Dudareva and Pichersky 2000; 288 Fischbach et al. 2002; Dudareva et al. 2004; van Schie et al. 2006). On the other hand, 289 some mechanisms that mediate and control terpene release (e.g. stomatal closure,

290 compound volatility and mechanisms of transport of terpenes across the cell) can 291 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 292 293 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 294 295 demonstrate a very high temperature-driven plasticity of plant physiological traits and 296 clearly emphasize the need to consider genotypic, epigenetic and phenotypic plasticity 297 in estimating and modeling floral emissions.

298 Our data demonstrate important variation in the temperature dependencies of 299 floral terpene emissions. In particular, the lower optimum temperatures for emission 300 maximum observed in species flowering in colder seasons and the higher optimum 301 temperatures observed in species flowering in warmer seasons indicate species-specific 302 temperature responses. This relationship suggests an adaptive mechanism that tunes 303 floral emissions to the temperatures to which the species are exposed during their 304 flowering season. Furthermore, our results also show this adaptive trend among 305 individuals of the same species, for example in D. viscosa, a species that has a long 306 flowering period and that was sampled in late summer and early autumn. This observed 307 seasonal change in the physiology of floral scent emission within a species indicates 308 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 309 310 in species with long flowering periods or with separate flowering periods throughout the 311 year to gain a more detailed insight into the intraspecific plasticity of the physiology of 312 flowers under different temperatures.

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Figure captions

Figure 1. Rates of total terpene emission per dry weight of floral tissue ($\mu g \ g \ DW^{-1} \ h^{-1}$) throughout the temperature gradient from 15 to 40 °C. The quantum flux density was maintained at 1000 μ mol 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).

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).





463 Figure 2



474 Figure 3

