



**HAL**  
open science

## Sources of floral scent variation in the food-deceptive orchid *Orchis mascula*

Laurent Dormont, Thomas Fort, Jean-Marie Bessière, Magali Proffit, Esther Garcia Hidalgo, Bruno Buatois, Bertrand Schatz

### ► To cite this version:

Laurent Dormont, Thomas Fort, Jean-Marie Bessière, Magali Proffit, Esther Garcia Hidalgo, et al.. Sources of floral scent variation in the food-deceptive orchid *Orchis mascula*. *Acta Oecologica*, 2020, 107, pp.103600. <10.1016/j.actao.2020.103600>. <hal-02995748>

**HAL Id: hal-02995748**

**<https://hal.science/hal-02995748v1>**

Submitted on 18 Jul 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC 4.0 - Attribution - Non-commercial use - International License

1 **Sources of floral scent variation in the food-deceptive orchid *Orchis***

2 ***mascula***

3

4

5 **Laurent Dormont<sup>1</sup>, Thomas Fort<sup>1</sup>, Jean-Marie Bessi re<sup>2</sup>, Magali Proffit<sup>1</sup>, Esther Garcia**

6 **Hidalgo<sup>1</sup>, Bruno Buatois<sup>1</sup> and Bertrand Schatz<sup>1</sup>**

7

8 <sup>1</sup>Centre d'Ecologie Fonctionnelle et Evolutive (CEFE), CNRS, 1919 Route de Mende, 34293

9 Montpellier, France

10 <sup>2</sup>Ecole Nationale Sup rieure de Chimie de Montpellier, Laboratoire de Chimie Appliqu e, 8

11 rue de l'Ecole Normale, 34296 Montpellier, France.

12

13

14 Corresponding author: [laurent.dormont@cefe.cnrs.fr](mailto:laurent.dormont@cefe.cnrs.fr)

15

16

17 **Abstract**

18 Intraspecific variation of floral signals, and particularly floral scent, is widespread in orchid  
19 species. Pollinator-mediated selection is often hypothesized to govern such polymorphism.  
20 For example, circadian rhythms of floral emissions have been associated to the daily activity  
21 patterns of pollinators in several plant species. However, most of the studies on floral scent  
22 variation usually consider only one factor of variation (diel variation, pollination, flower  
23 age...). In this study, we investigated simultaneously seven sources of variation susceptible to  
24 induce changes in floral scent emissions in the food-deceptive orchid *Orchis mascula*:  
25 circadian rhythm, flower age, pollination, inflorescence morphology, herbivory, leaf spots and  
26 habitat. We found that all the factors studied, except plant morphology (spots on leaves,  
27 inflorescence morphology), were associated with significant changes in the volatile profile of  
28 *O. mascula* inflorescences. Electroantennography experiments with *Bombus terrestris*, the  
29 main pollinator of *O. mascula*, revealed that bumblebees were able to detect almost all the  
30 compounds involved in floral scent variation. Whether pollinator behaviour is influenced by  
31 changes in floral odours induced by these different factors, and whether it can affect plant  
32 reproductive success will have to be tested in further studies.

33

34 **Keywords:** *Orchis mascula*, floral scent, intraspecific variation, plant-pollinator interactions

35

36

## 37 **Introduction**

38

39         Odour emitted by flowers represents a key floral signal used by insects to detect and  
40 select rewarding flower species (Raguso, 2008; Schiestl, 2015). Most flower-visitor species  
41 orient their flight behaviour by exploiting single volatile compounds or complex volatile  
42 blends provided by flowers (Bruce et al., 2005). In many plant species, chemical profiles of  
43 floral scents have been reported to vary both within and among individuals as well as among  
44 populations (Knudsen et al., 2006; Delle-Vedove et al., 2017). Why does floral scent vary  
45 within a plant species has long intrigued ecologists and has generated many research studies?  
46 Several of them have highlighted the key role of pollinators in driving chemical variation  
47 between conspecific populations, or even closely related species, through pollinator-mediated  
48 selection (Hoballah et al., 2007; Raguso 2008; Suchet et al., 2011). Several factors other than  
49 pollinators have also been proposed to explain intraspecific variation of floral scent, such as  
50 antagonistic interactions with herbivores and pathogens, genetic drift or phenotypic plasticity  
51 (Majetic et al., 2008, 2009; Schlumpberger and Raguso, 2008). Other authors have  
52 investigated floral odour variation in relation to floral colour variation, and found that distinct  
53 colour morphs in a given plant species differ in scent chemistry (Odell et al., 1999; Flamini et  
54 al., 2002; Zucker et al., 2002; Li et al., 2006; Salzman and Schiestl, 2007). Floral colour-  
55 odour associations have been proposed to result from biochemical processes that affect both  
56 traits, because pigments and volatile compounds often share common biosynthetic pathways  
57 (Zucker et al., 2002; Majetic et al., 2007; Delle-Vedove et al., 2011).

58         Beyond the direct key influence of pollinators on floral scent variation, several other  
59 sources of spatial and temporal variation in floral scent emissions have been reported (Raguso  
60 and Weiss 2015; Delle-Vedove et al., 2017). For examples, intraspecific changes in floral  
61 volatiles have been observed in relation with pollination (Tollsten and Bergström, 1989;

62 Negre et al, 2003; Theis and Raguso, 2005; Muhlemann et al., 2006; Proffit et al., 2008),  
63 circadian rhythms (Kolossova et al., 2001; Raguso et al., 2003; Theis et al., 2007; Okamoto et  
64 al., 2008; Fenske and Imaizumi, 2016; Chapurlat et al., 2018, 2019), herbivory (Kessler and  
65 Halitschke, 2009; Kessler et al., 2010, 2013; Gish et al., 2015), flower age (Schade et al.,  
66 2001; Steenhuisen et al., 2010; Filella et al, 2013; Wang et al., 2018), or habitat origin  
67 (Majetic et al., 2009; D'Auria et al., 2019). However, very few studies have examined the  
68 combined effects of these distinct sources of variation. How these factors may successively or  
69 simultaneously act on floral volatiles within a single plant species (and even within one  
70 population), and what are the relative influences of these different factors remains largely  
71 unknown.

72

73         The orchid family represents a very interesting case of floral polymorphism, which is  
74 generally associated with animal pollination (Van der Cingel, 1995; Claessens and Kleynen,  
75 2011). Approximately one-third of all orchid species achieve pollination through food  
76 deception, *i.e.*, flowers contain no nectar or other rewards but resemble or mimic floral signals  
77 of rewarding plants to attract pollinators (Jersakova et al., 2006). Intraspecific variation in  
78 floral traits, and particularly odours, is expected to be high in food-deceptive orchids, because  
79 flowers must delay the avoidance learning of pollinators (Jersakova et al., 2006; Schiestl,  
80 2005). Many orchids exhibit such odor polymorphism, both rewarding and deceptive species  
81 (Tollsten and Bergström, 1989; Moya and Ackerman, 1993; Olesen and Knudsen, 1994;  
82 Azuma et al., 2001; Dötterl et al., 2005; Salzman et al., 2007; Dormont et al., 2014, 2019).  
83 However, most of these studies have analyzed floral scent by performing a single sampling  
84 session, and did not considered other possible sources of odor variation. In fact, as mentioned  
85 above, intraspecific variation of floral scent may occur under the influence of various biotic or  
86 abiotic factors.

87           In this study, we investigated simultaneously the effects of different factors on the  
88 floral scent of the food-deceptive orchid *Orchis mascula* L. This species is distributed  
89 throughout Europe, and typically exhibits red-purple flowers, except some populations where  
90 rare white-flowered individuals can be observed within purple-flowered patches. Floral  
91 volatiles of these two-colour morphs have been investigated in previous studies (Dormont et  
92 al., 2010, 2014), and revealed no differences of volatile profile between the two-colour  
93 morphs. The objective of this study was to examine whether the floral scent emissions of *O.*  
94 *mascula* are affected by different biotic or abiotic conditions: flower age, circadian rhythm,  
95 plant morphology (number of flowers per inflorescence, spots on leaves), plant habitat,  
96 pollination, or herbivore attacks. All these factors were investigated for the same populations  
97 and at the same period, allowing to estimate different causes of variations and their compared  
98 amplitude for same individuals in a same context. We also investigated the  
99 electrophysiological responses of naïve bumblebees, the most frequent pollinators of *O.*  
100 *mascula* flowers, to relevant identified volatile organic compounds which were found to vary  
101 under the influence of these factors.

102

## 103 **Material and methods**

104

### 105 **Study sites and organism**

106           The experiments were carried out in two different sites, separated by 3.5 km, in a  
107 mountainous area of south-central France. Both sites were located about 70 km north of  
108 Montpellier, on the Causse du Blandas, an extensive limestone plateau (43°55' N, 3°29' E,  
109 710 m altitude). In each site, we surveyed several patches of *O. mascula* individuals (8  
110 patches in site 1, and 8 patches in site 2), each of which consisted of about 20 to 100  
111 individuals and was separated by at least 50 m (usually 300 m or more) from the nearest

112 neighbouring patch. In this study, and because most patches were very distant from each  
113 other, each patch of 20-100 individuals was considered as a “population”. The first site  
114 consisted of an open dry grassland, while populations of the second site were situated in the  
115 undergrowth of pine wood.

116 The species *O. mascula* is a perennial non-rewarding orchid species, widely  
117 distributed in Europe, western Asia, and northern Africa, where it is present from 0 to 2500m.  
118 This species is usually present in open habitats, but it is also shade tolerant (Jacquemyn et al.,  
119 2009b). Inflorescences consist generally of 5 to 20 typically purple flowers. In some  
120 populations, a few white-flowered individuals (Dormont et al., 2010) and pink-flowered  
121 individuals (Schatz et al., 2013) occur mixed with the purple-flowered individuals. *O.*  
122 *mascula* flowers are pollinator-dependent for setting seeds (Nilsson, 1983; B. Schatz,  
123 unpublished), and are visited and pollinated by bumblebees (*Bombus* spp.) or honeybees,  
124 cuckoo bumblebees (*Psithyrus* spp.) and *Nomada* sp., solitary bee species of several genera  
125 (*Eucera*, *Nomada*, *Andrena*, *Halictus* and *Osmia*), a chafer beetle (*Cetonia aurata*) and more  
126 anecdotally by one diptera (*Bombylius major*) and one lepidoptera (*Zygaena transalpina*)  
127 (Nilsson, 1983; Bournérias and Prat, 2005; Cozzolino et al., 2005; Jacquemyn et al., 2009b;  
128 Joffard et al., 2019). Flowering occurs early in spring, and *O. mascula* is known to exploit  
129 newly emerged insect pollinators, suggesting that pollination in this species is performed  
130 mainly by visits of naïve, inexperienced insects (Nilsson, 1983; Van der Cingel, 1995). Adult  
131 *O. mascula* plants are known to show a pronounced small-scale aggregation, because  
132 seedlings are clustered around adult plants, with approximately 90% of all recruits located  
133 within 16 and 24 cm of the adults in populations (Jacquemyn et al., 2009a, 2009b).

134

## 135 **Floral volatiles**

136 *Sampling of floral volatiles*

137 Floral volatiles were monitored using solid-phase microextraction (SPME), a non-  
138 destructive, solvent-free sampling technique permitting the sampling of volatiles in situ on  
139 living plant individuals. Sampling by SPME was performed using 65  $\mu\text{m}$   
140 polydimethylsiloxane / divinyl benzene (PDMS-DVB) fibres (Supelco®). The whole  
141 inflorescence was enclosed in a bag made from polyethylene terephthalate (Nalophan®; Kalle  
142 Nalo GmbH, Würsthüllen, Germany), a nonreactive plastic. After the equilibration time (15  
143 min), the fibre was introduced with a manual holder into the Nalophan® bag containing the  
144 inflorescence. The fibre was exposed for 45 min in close proximity (1 cm) to flowers. For  
145 each sampled population of *O. mascula* individuals, a control bag was also sampled: an  
146 SPME fibre was inserted into an empty Nalophan bag, in order to monitor volatiles from the  
147 air surrounding the plant.

148 Most of the time, the period of floral volatile sampling in situ was defined with respect  
149 to both the flowering period (April 2014) and the period of maximum activity of insects  
150 during the day, *i.e.*, between 11:00 and 15:00. We explored different possible sources of floral  
151 scent variations by carrying volatile sampling under different situations. For each situation, a  
152 particular attention was given to sample individuals under the influence of only one factor of  
153 variation. For example, when studying the possible effects of habitat, inflorescences were  
154 sampled among plants that were non-pollinated (assessed by a rigorous visual check of each  
155 flower before sampling), showing no herbivore attack, and sampling was performed at 13.00  
156 on individuals at mid-flowering period.

157 - Habitat: 20 *O. mascula* individuals were randomly selected and sampled for scent  
158 collection in the first site (open dry grassland), and 20 other individuals were sampled  
159 in the second site (pine wood).

- 160 - Circadian rhythm: odour emissions from 20 *O. mascula* individuals, with a similar  
161 number of flowers per inflorescence, of the first site were sampled at different times of  
162 the day, at 9.00-10.30 a.m., at 13.30-15.00 p.m, and 18.00-19.30 p.m.
- 163 - Flower age: the same 20 individuals were sampled at different periods of flowering, at  
164 1, 6 and 13 days after blooming. Individuals were surveyed every day before blooming  
165 to determine the first day of sampling.
- 166 - Inflorescence morphology: Four categories of individuals were defined, with respect  
167 to the number of flowers per inflorescence (less than 5 flowers, 6-11 flowers, 12-20  
168 flowers, more than 21 flowers). 15 individuals of each category, *i.e.* of different  
169 inflorescence size, were sampled as described above.
- 170 - Spots on leaves: some *O. mascula* individuals exhibit dark spots on the basal leaves,  
171 while other individuals have no spots. We sampled both types of individuals, 20  
172 individuals of each type.
- 173 - Pollination: odours from 20 *O. mascula* individuals were sampled 2 days after  
174 blooming, were then left during 8 days until manual pollination of the flowers was  
175 performed, and odours were then sampled again two days after pollination. 10 other  
176 “control” individuals were sampled similarly as above, but were not manually  
177 pollinated and inflorescences were protected with bags until second odour collection.
- 178 - Herbivory: 20 other *O. mascula* individuals of the first site were randomly selected  
179 and volatile emissions were sampled on day 3 after blooming. Insect flower damage  
180 was simulated by cutting both a piece of leaf (total length of 10 cm cutting) and  
181 labellum extremity (half of the flowers from each inflorescence was treated). Odour  
182 sampling was performed two days after artificial damage, to avoid isolation of  
183 possible damage-related volatile compounds immediately after damage.

184

185 Overall, a total of 162 *O. mascula* inflorescences (162 different individuals) were sampled for  
186 floral scent during this study.

187

### 188 *Gas chromatography-mass spectrometry of floral volatiles*

189 GC-MS analyses of the SPME extracts were performed using electronic impact  
190 ionization mode on a Varian Saturn 2000 ion trap spectrometer, interfaced with a Varian GC  
191 CP-3800 apparatus. The Varian CP-3800 was equipped with a 1079 split-splitless injector  
192 (260 °C) and a 30 m x 0.25 mm x 0.25 µm film thickness ID WCOT CPSil 8CB fused silica  
193 capillary column (Chrompack®, Bergen op Zoom, The Netherlands), with helium as carrier  
194 gas (1 ml min<sup>-1</sup>), and programmed 2 min isothermal at 50 °C, then 50 °C to 220 °C at 4 °C  
195 min<sup>-1</sup>. Mass spectra were recorded in electronic impact (EI) at 70eV, and identified by  
196 comparison with data of the NIST 98 software library. Floral volatile compounds were  
197 identified based on retention time of external synthetic standards, and with mass spectra  
198 analyses of GC-MS data. Peaks were quantified using Star Chromatography Software®. The  
199 relative importance of each compound was expressed with respect to total volatiles in order to  
200 compare the volatile profile of the samples.

201

### 202 **Electroantennography assays**

203 Electroantennography assays were performed using a commercial colony of naïve  
204 *Bombus terrestris*. This species is known to be among the main pollinators of *O. mascula* in  
205 southern France (Nilsson 1983; Joffard et al., 2019; Schatz B., unpublished data). The colony  
206 was purchased from BioBest Group NV® (Westerlo, Belgium). Only female workers were  
207 used in our tests. Each female bumblebee was immobilized 5 minutes at 3°C. The head was  
208 cut and separated from the thorax, and put into a glass capillary (L: 76 mm, Ø: 1.12 mm)  
209 (World Precision Instrument, Sarasota, USA) pulled and cut using a vertical micropipette-

210 puller (P-30 model, World Precision Instruments, Hertfordshire, United Kingdom). The  
211 capillary was previously filled with an electrolytic solution of ringer (8.0 gl<sup>-1</sup> NaCl, 0.4 gl<sup>-1</sup>  
212 KCl, 0.4 gl<sup>-1</sup> CaCl<sub>2</sub>) and connected to a reference electrode. The tip of one antenna was  
213 connected into the recording electrode, also filled with the electrolytic solution, at the  
214 opposite side of the reference electrode. A continuous flow of purified and humidified air,  
215 passing through a glass tube to the antenna at a flow rate of 330 ml min<sup>-1</sup>, was provided by a  
216 stimulus controller (CS-55 Syntech, Hilversum, The Netherlands). The antenna was then  
217 stimulated with a single compound, and antennal electrical responses to volatile compounds  
218 were measured for 31 female bumblebees and digitized using an IDAC board (acquisition  
219 controller IDAC-2; Syntech, Hilversum, the Netherlands) and data was processed with a PC-  
220 based interface and software package (GcEad 1.2.5, Syntech). Sixteen synthetic volatile  
221 compounds, which were identified in the floral scents of *O. mascula*, were tested. These  
222 compounds were the main components of *O. mascula* floral scent, and included the  
223 compounds that were found to vary during this study: linalool, limonene,  $\alpha$ -pinene, 6-methyl-  
224 5-hepten-2-one, linalool-oxide, eucalyptol, myrcene, methyl-decanoate,  $\beta$ -farnesene, 4-  
225 phenyl-2-butanone, methyl-octanoate, terpinyl-acetate,  $\beta$ -pinene, (Z)-3-hexenyl-acetate,  
226 ocimene, methyl-cinnamate (Sigma-Aldrich®, US, Bellefonte; TCI Chemical®, Stockholm,  
227 Sweden; and Extrasynthese®, Genay, France).

228 Each synthetic compound was individually tested at different dilutions, solutions being made  
229 at 0.1%, 1%, and 10% v/v in dichloromethane. For each test, 1  $\mu$ l of pure solution was  
230 deposited on a strip of filter paper and left for evaporation during 5 min in ambient conditions  
231 in a separate laboratory room. The antenna was stimulated with pulses of 0.5 s each, using a  
232 purified and moistened airflow of 11.3 mL s<sup>-1</sup> across a Pasteur pipette containing the filter  
233 paper. Each of the sixteen compounds was delivered once for each dilution; compounds were  
234 presented in the same order so that all the antennae were submitted to a similar stimulus

235 sequence, thus allowing comparisons between antennal signals. Stimuli were separated by a  
236 40 s interval, to avoid saturation of the olfactory receptors. A control was performed at the  
237 beginning of each experimental series by measuring the antenna response to clean air and to 1  
238  $\mu$ l of dichloromethane deposited on a filter paper. For each bumblebee antenna, the response  
239 amplitude was normalized to the maximum response recorded.

240

## 241 **Data analysis**

242 The chemical composition of floral volatiles was analysed in R 2.14.1 (R  
243 Development Core Team, 2011) using multivariate analysis incorporated in the Vegan  
244 package (Oksanen et al., 2012). For each comparison, the relative proportion of each  
245 compound that accounted for more than 1% of total volatiles was included in the analyses.  
246 Following Soler et al. (2012), the data were first transformed using squared root and  
247 Wisconsin double standardization, and then a data matrix of pairwise Bray-Curtis  
248 dissimilarity indices between samples was build. Secondly, Permutational Multivariate  
249 Analysis of Variance (PERMANOVA) on the distance matrices based on 999 permutations  
250 (Anderson, 2001) were performed to test for significant differences in the floral scent  
251 composition among different situations. In addition, a multivariate Levene's test was  
252 performed to test the homogeneity of group variances for each factor. When factors were  
253 observed to affect significantly orchid volatiles, a similarity percentage (SIMPER) was used  
254 to identify the compounds responsible for more than 30 % of the variation.

255 To determinate the antennal response to each compound in electroantennography  
256 assays, we considered the amplitude of depolarization induced by the stimulus compared to  
257 the corresponding baseline, and significance of the responses were tested by using Paired t-  
258 tests as described by Vittecoq et al. (2011).

259

## 260 **Results**

### 261 **General composition of floral volatiles**

262 A total of 92 volatile compounds were isolated from the inflorescences of *O. mascula*  
263 when considering the different samplings (Table 1). The volatile profile was largely  
264 dominated by terpene components: 63 of the 92 identified compounds (68%) originated from  
265 the terpenoid biosynthetic pathway. Among the twelve most abundant compounds, eleven are  
266 terpenes; the cumulated value of relative proportion of all compounds is 19.56%. The  
267 chemical composition of the floral scent was often dominated by one terpene product,  
268 limonene, which accounted for a mean of 25.5 % of floral volatiles. Six other compounds  
269 were predominant (>5% of the profile) in the floral scent of *O. mascula*: (E)-ocimene (8.8%),  
270 linalool (7.7%),  $\alpha$ -pinene (6.6%), myrcene (6.3%), 6-methyl-5-hepten-2-one (5.6%), and  
271 linalool-oxide (5.3%).

272

### 273 **Variation of floral volatiles**

274 Among the possible sources of scent variation explored in this study, five  
275 environmental and biological factors (habitat, flower age, circadian rhythm, pollination,  
276 herbivory) were associated with a significant modification of volatile profiles (Table 2, Figure  
277 1). Variation always consisted of changes in the proportion of some volatile compounds, no  
278 qualitative changes were observed for these factors. The two last factors related to  
279 morphology (number of flowers, spots on leaves) had no significant influence on floral scent  
280 composition.

281

#### 282 *Floral scent variation and habitat*

283 The dissimilarity analysis showed that habitat had a substantial effect on the variation  
284 in floral volatiles (PERMANOVA  $F_{1,38} = 3.386$ ;  $p < 0.001$ ). Comparison of the floral volatile

285 profiles between open and closed habitats was characterized by an increase proportion of  
286 some terpenes (myrcene,  $\beta$ -pinene, eucalyptol) in the woody pine site, while other compounds  
287 were observed to decrease in this closed habitat (limonene, sabinene hydrate, linalool oxide)  
288 (Table 1).

289

#### 290 *Floral scent variation and flower age*

291 Modification of floral volatile profiles was particularly great in relation with flower  
292 age (PERMANOVA  $F_{1,38} = 3.386$ ;  $p=0.001$ ). Difference in the variance among groups ( $F_{1,38}$   
293  $= 5.472$ ;  $p = 0.007$ ) may partly explain this variation. Several compounds were found to vary  
294 in their emission over the period of flowering, such as for limonene or 3-hexenyl acetate,  
295 which decreased with flower age, or for 6-methyl-5-hepten-2-one and eucalyptol, which both  
296 increased at the end of the flowering period.

297

#### 298 *Floral scent variation and circadian rhythm*

299 Floral volatiles were observed to vary also with circadian rhythm (PERMANOVA  $F$   
300  $_{2,52} = 2.393$ ;  $p<0.001$ ). Modification of floral emissions was particularly significant for  $\beta$ -  
301 pinene, and p-cymene, which increased over the day.

302

#### 303 *Floral scent variation and pollination*

304 A significant relationship was also observed between floral emissions and pollination:  
305 floral samplings before and after pollination revealed a modification of volatile profiles  
306 (PERMANOVA  $F_{1,28} = 3.806$ ;  $p<0.001$ ). Several volatile compounds showed a drastic change  
307 in their emission after pollination. In particular, the floral emissions were characterized by a  
308 significant increase of the proportion of  $\alpha$ -pinene, myrcene, 6-methyl-5-hepten-2-one and

309 eucalyptol, while the proportions of other compounds were lower after pollination, such as for  
310 *Z*-ocimene and linalool.

311

### 312 *Floral scent variation and herbivory*

313 Herbivory, which was simulated by damaging both leaves and labellum, also resulted  
314 in a significant change of floral volatiles (PERMANOVA  $F_{1,28} = 3.006$ ;  $p < 0.001$ ). Artificial  
315 herbivore damage was followed by increased proportions of sabinene, myrcene, eucalyptol,  
316 and ocimene, while the levels of other compounds (limonene, 3-hexenyl acetate) were lower  
317 after damage.

318

### 319 **Antennal detection of synthetic compounds**

320 All synthetic compounds were detected by antennae and elicited strong EAG  
321 responses at the different dilutions tested (Fig. 2). Tests with clean air or control samples  
322 (dichloromethane) had no effect on antennal responses. The highest amplitude of  
323 depolarization was recorded for linalool.  $\alpha$ -pinene and  $\beta$ -pinene induced a significant  
324 response from the antenna only for 10% dilutions, but no significant electric responses could  
325 be recorded for 0.1% or 1% solutions.

326

### 327 **Discussion**

328 Our study demonstrated that floral scent emissions of *O. mascula* can vary under the  
329 influence of several biotic or abiotic factors: while plant morphology (spots on leaves,  
330 inflorescence size) had no effect on floral volatiles, all the other factors (flower age, circadian  
331 rhythm, pollination, habitat, herbivory) were associated with significant changes in the  
332 volatile profile of *O. mascula* inflorescences. The most important effects were recorded for  
333 flower age and pollination, although a possible inherent confounding effect may occur

334 between these two factors: for a few volatile compounds, variation of compound levels after  
335 pollination might also partly results from the effect of flower age. Modification of the floral  
336 scent induced by these different factors always consisted of changes in the ratio of a few  
337 number of volatile compounds, mostly monoterpenes. No qualitative changes of the floral  
338 scent composition were induced by these factors.

339 Many other studies have already investigated the effects of similar factors on floral  
340 scent profile, but in most case experiments were limited to the analysis of only one or two  
341 factor(s) of variation. Significant changes in the floral emissions were found for most of the  
342 factors studied. For example, studies that examined how floral scent may vary after  
343 pollination reported substantial variation of the volatile profile, consisting mainly of  
344 modification in the relative amounts of volatile compounds observed to have a key-role for  
345 pollinator attraction (Schiestl and Ayasse, 2001; Theis and Raguso, 2005; Muhlemann et al.,  
346 2006). Diel variation in floral scent has also been reported for number of plants, including  
347 orchid species (Nilsson, 1978; Chapurlat et al., 2018; Steen et al., 2019). Numerous examples  
348 of temporal variation in floral scent through floral development from anthesis to senescence  
349 have also been provided from literature (Schade et al., 2001; Steenhuisen et al., 2010; Filella  
350 et al., 2013; Wang et al., 2018). Similarly, changes in floral scent patterns following herbivore  
351 attacks have been described in many plant families (Kessler and Halitschke, 2009; Kessler et  
352 al., 2010, 2013; Gish et al., 2015). Finally, other authors have also observed intraspecific  
353 variation in floral scent in relation to geographic origin or population membership (Svensson  
354 et al., 2005; Majetic et al., 2008; Schlumpberger and Raguso, 2008; D'Auria et al., 2019).

355 All these studies focused on one factor likely responsible for floral scent variation,  
356 whereas we tentatively investigated in this study the different sources of changes in floral  
357 emissions within a single meta-population of *O. mascula*. Such a sampling allowed to  
358 compare causes of variations on same individuals, and thus help understanding the relative

359 importance of different factors. Our results confirmed that these different factors have  
360 individually a drastic effect on floral scent emissions, which likely explains the high level of  
361 intraspecific variation of *O. mascula* scent already reported in literature (Nilsson, 1983;  
362 Salzmann et al., 2007; Jacquemyn et al 2009b; Dormont et al., 2010, 2014). We showed that  
363 several factors may act simultaneously on the floral scent emissions. Interestingly, different  
364 compounds were observed to vary, according to the source of variation. However, variation  
365 was mostly recorded for compounds that were both dominant in the profile (>3%) and  
366 frequent in the samples (occurrence > 100 of the 162 samples).

367         Two main questions emerge from these results on *O. mascula*: (i) Are insects  
368 (pollinators, visitors, or herbivores) capable to detect such variation in floral scent, and does  
369 this variation have consequences on pollinator behaviour? And (ii) Why do the floral  
370 emissions vary? Many insects, and particularly pollinator species, are known to respond to  
371 minor changes in the odour profile of their host plant (Wright et al., 2005; Lawson et al.,  
372 2018). Modifying the specific ratio of few compounds in a volatile blend usually results in  
373 altering the behaviour of insects (Bruce et al., 2005, Bruce and Pickett, 2011; Tasin et al.,  
374 2005; Cha et al., 2011; Salvagnin et al., 2018), so that small changes in the volatile profile  
375 may have drastic consequence for pollinator choice and plant pollination. In our case, since  
376 we did not examine the olfactory responses of pollinators to such changes in *O. mascula*  
377 volatiles, we cannot assume that variation in the scent profile have consequences on pollinator  
378 behaviour nor on plant reproductive success. However, most of the compounds that were  
379 shown to vary in our study (myrcene, 6-methyl-5-hepten-2-one, eucalyptol, ocimene, linalool,  
380 linalool oxide, limonene, 3-hexenyl acetate...) were detected by bumblebees during  
381 electroantennography assays, as well as in previous studies (Suchet et al., 2011). These  
382 compounds have also been reported to elicit EAG responses for other *O. mascula* pollinator  
383 species, such as for *Cetonia aurata* (Steenhuisen et al., 2013) or *Apis mellifera* (Henning et

384 al., 1992). Further studies will thus have to test, using the main pollinator species of *O.*  
385 *mascula*, whether variation of floral scent such as those we observed during this study,  
386 actually influences pollinator behaviour.

387 Another question that remains unanswered is why does floral scent vary? Although  
388 several hypotheses have been proposed, current explanations for polymorphism in floral  
389 odours most frequently rely on the key role of insects through pollinator-mediated selection  
390 (Delle-Vedove et al., 2017). In our case, a change in the floral signal over flower phenology  
391 (flower age) and after pollination likely helps *O. mascula* to guide pollinators to receptive  
392 flowers and to avoid visitations to already pollinated flowers, respectively. Diel variation of  
393 floral emissions may synchronize the floral attractive scent with circadian rhythms of  
394 particular pollinators, such as it has been shown in several plant species (Hoballah et al.,  
395 2005; Farré-Armengol et al., 2013; Prieto-Benitez et al., 2016; Chapurlat et al., 2018; Fenske  
396 et al., 2018; Steen et al., 2019).

397 Regarding changes in *O. mascula* scent following artificial herbivory, it is well  
398 established that plants being attacked by herbivores start releasing particular volatile  
399 compounds, so-called “herbivore-induced plant volatiles” (Dicke and Baldwin, 2010). Floral  
400 scent has also been shown to vary under herbivore attacks (Lucas-Barbosa et al., 2011). While  
401 such volatile emissions have often been considered to have a defensive function (Turlings and  
402 Erb, 2018), *e.g.* by attracting predators and parasitoids, the ecological significance and  
403 evolution of herbivore-induced plant volatiles are still under debate (Dicke and Baldwin,  
404 2010; Turlings and Erb, 2018).

405 Floral emissions were also observed to vary among populations from different habitats  
406 (open grassland vs. pine wood site) in our study. Intraspecific variation in floral scent in  
407 relation to population membership or even geographic origin have often been associated with  
408 distinct pollinator guilds (Sun et al., 2013; Peter and Johnson, 2013), but the effect of other

409 environmental factors or the occurrence of phenotypic plasticity have been also reported  
410 (Majetic et al., 2008, 2009; Schlumpberger and Raguso, 2008; D'Auria et al., 2019).

411 Interestingly, inflorescence morphology in our study (comparison of four categories of  
412 inflorescence size, defined by the number of flowers) was not associated with a change in  
413 floral scent. However, it should be note that the total amount of scent emitted by large  
414 inflorescences (with more flowers) may be greater than smaller inflorescences: we did not  
415 examine this possible effect on pollinator attraction in our study. Variation of this floral trait,  
416 which is associated with a change in floral display, has been reported to have a drastic effect  
417 on plant fruit set in rewardless orchids (Pellegrino et al., 2010; Suetsugu et al., 2015). It has  
418 been suggested that variation in inflorescence size may have more influence on pollinator  
419 attraction than changes in floral scent in some cases (Kindlmann and Jersáková, 2006).

420 All these examples suggest the key role of insects in floral scent variation: pollinator-  
421 mediated selection likely explain numerous situations of polymorphism in floral emissions. In  
422 *O. mascula*, the exact role of pollinators in floral scent variation remains unknown, because  
423 behavioural responses of insects to varying floral emissions was not examined in our study. It  
424 has also been proposed that variation in floral scent may also result from the influence of  
425 natural enemies, non-selective agents, or even environmental factors (Majetic et al., 2009;  
426 Raguso et al., 2015; Delle-Vedove et al., 2017). Such factors will have to be considered in  
427 further studies in *O. mascula*.

428 Our study showed that floral scent may vary, successively or simultaneously, under  
429 the influence of various factors. Different compounds are involved in floral scent variation,  
430 depending on the factor considered. To what extent pollinators may drive such variation, and  
431 how these changes in floral emissions affect plant reproductive success remain to be  
432 determined. For example, further olfactory tests that examine behavioural responses of

433 different pollinator species to varying *O. mascula* volatiles, are strongly needed to further  
434 evaluate the role of pollinator-mediated selection in intraspecific variation of floral scent.

435

#### 436 **Acknowledgments**

437 We thank Simon Chollet, Catherine Soler, and Jean-Dominique Lebreton for help in  
438 experimental design, and Camille Guillem for comments on the manuscript. We also thank  
439 the GDR MediatEC N°3658 CNRS and the GDR Pollineco n°2058 CNRS-MTES for useful  
440 discussion.

441

#### 442 **Author contribution**

443 LD, BS, and TF designed the study. LD, BS, TF, and EGH conducted the experiments in the  
444 field. TF, BB, and EGH performed EAG experiments. BB and JMB analysed gas  
445 chromatography/mass spectrometry data. MP performed statistical analyses. LD, BS, MP  
446 drafted the manuscript. All authors contributed with revisions and approved the final  
447 manuscript.

448

449

#### 450 **References**

- 451 Anderson, M.J., 2001. Permutation tests for univariate or multivariate analysis of variance and  
452 regression. *Can. J. Fish. Aquat. Sci.* 58: 626-639.
- 453 Azuma, H., Toyota, M., Asakawa, Y., 2001. Intraspecific variation of floral scent chemistry in  
454 *Magnolia kobus* DC. (Magnoliaceae). *J. Plant Res.* 114: 411-422.
- 455 Bournérias, M., Prat, D., 2005. Les orchidées de France, Belgique et Luxembourg : deuxième  
456 édition. Biotope, Mèze, France.
- 457 Bruce, T.J., Wadhams, L.J., Woodcock, C.M., 2005. Insect host location: a volatile situation.  
458 *Trends in Plant Sci.* 10, 269-274.
- 459 Bruce, T.J.A., Pickett, J.A., 2011. Perception of plant volatile blends by herbivorous insects -  
460 finding the right mix. *Phytochem.* 72, 1605-1611.

461 Cha, D.H., Linn Jr, C.E., Teal, P.E., Zhang, A., Roelofs, W.L., Loeb, G.M., 2011.  
462 Eavesdropping on plant volatiles by a specialist moth: significance of ratio and  
463 concentration. PLoS One 6, e17033.

464 Chapurlat, E., Ågren, J., Anderson, J., Friberg, M., Sletvold, N., 2019. Conflicting selection  
465 on floral scent emission in the orchid *Gymnadenia conopsea*. New Phytol. 222, 2009-  
466 2022.

467 Chapurlat, E., Anderson, J., Ågren, J., Friberg, M., Sletvold, N., 2018. Diel pattern of floral  
468 scent emission matches the relative importance of diurnal and nocturnal pollinators in  
469 populations of *Gymnadenia conopsea*. Ann. Bot. 121, 711-721.

470 Claessens, J., Kleynen, J., 2011. The flower of the European orchid: Form and function. Jean  
471 Claessens & Jacques Kleynen, Geulle, 439 p.

472 van der Cingel, N.A., 1995. An atlas of orchid pollination-European orchids. Balkema,  
473 Rotterdam, 192 p.

474 Cozzolino, S., Schiestl, F.P., Muller, A., De Castro, O., Nardella, A.M., Widmer, A., 2005.  
475 Evidence for pollinator sharing in Mediterranean nectar-mimic orchids: Absence of  
476 pre-mating barriers? Proc. Roy. Soc. B: Biol. Sci. 272, 1271–1278.

477 D'Auria, M., Lorenz, R., Mecca, M., Racioppi, R., Antonio Romano, V., Viggiani, L., 2019.  
478 Fragrance components of *Platanthera bifolia* subsp. *osca* and *Platanthera chlorantha*  
479 collected in several sites in Italy. Nat. Prod. Res. 1-5.

480 Delle-Vedove, R., Schatz, B., Dufayé, M., 2017. Understanding intraspecific variation of floral  
481 scents in the light of evolutionary ecology. Ann. Bot. 120, 1-20.

482 Delle-Vedove, R., Juillet, N., Bessière, J.M., Grison, C., Barthes, N., Pailler, T., Dormont, L.,  
483 Schatz, B. 2011. Colour-scent associations in a tropical orchid: three colours but two  
484 odours. Phytochem. 72, 735-742.

485 Dicke, M., Baldwin, I.T. 2010. The evolutionary context for herbivore-induced plant  
486 volatiles: beyond the 'cry for help'. Trends Plant Sci. 15, 167-175.

487 Dormont, L., Delle-Vedove, R., Bessière, J.-M., Hossaert-McKey, M., Schatz, B. 2010. Rare  
488 white-flowered morphs increase the reproductive success of common purple morphs in a  
489 food-deceptive orchid. New Phytol. 185, 300-310.

490 Dormont, L., Delle-Vedove, R., Bessière, J.M., Schatz, B., 2014. Floral scent emitted by  
491 white and colored morphs in orchids. Phytochem. 100, 51-59.

492 Dormont, L., Joffard, N., Schatz, B., 2019. Intraspecific variation in floral color and odor in  
493 orchids. Int. J. Plant Sci. 180(9):1036–1058.

494 Dötterl, S., Wolfe, L.M., Jürgens, A, 2005. Qualitative and quantitative analyses of flower  
495 scent in *Silene latifolia*. *Phytochem.* 66: 203–213.

496 Farré-Armengol, G., Filella, I., Llusia, J., Penuelas, J., 2013. Floral volatile organic  
497 compounds: between attraction and deterrence of visitors under global change. *Persp.*  
498 *Plant Ecol. Evol. Syst.*, 15, 56-67.

499 Fenske, M.P., Imaizumi, T., 2016. Circadian rhythms in floral scent emission. *Front. Plant*  
500 *Sci.*, 7: 462.

501 Fenske, M.P., Nguyen, L.P., Horn, E.K., Riffell, J.A., Imaizumi, T., 2018. Circadian clocks of  
502 both plants and pollinators influence flower seeking behavior of the pollinator hawkmoth  
503 *Manduca sexta*. *Sci. Rep.*, 8: 2842.

504 Filella, I., Primante, C., Llusia, J., González, A.M.M., Seco, R., Farré-Armengol, G., Rodrigo,  
505 A., Bosch, J., Penuelas, J., 2013. Floral advertisement scent in a changing plant-  
506 pollinators market. *Sci. Rep.*, 3: 3434.

507 Flamini, G., Cioni, P.L., Morelli, I., 2002. Analysis of the essential oil of the aerial parts of  
508 *Viola etrusca* from Monte Labbro (South Tuscany, Italy) and in vivo analysis of flower  
509 volatiles using SPME. *Flav. Fragr. J.*, 17: 147–149.

510 Gish, M., De Moraes, C.M., Mescher, M.C., 2015. Herbivore-induced plant volatiles in  
511 natural and agricultural ecosystems: open questions and future prospects. *Curr. Opin.*  
512 *Insect Sci.*, 9: 1-6.

513 Granero, A.M., Sanz, J.M.G., Gonzalez, F.J.E., Vidal, J.L.M., Dornhaus, A., Ghani, J.,  
514 Serrano, A.R., Chittka, L. 2005. Chemical compounds of the foraging recruitment  
515 pheromone in bumblebees. *Naturwissenschaften* 92, 371–374. Henning, J.A., Peng, Y.S.,  
516 Montague, M.A., Teuber, L.R., 1992. Honey bee (Hymenoptera: Apidae) behavioral  
517 response to primary alfalfa (Rosales: Fabaceae) floral volatiles. *J. Econ. Entomol.* 85:  
518 233-239.

519 Hoballah, M.E., Stuurman, J., Turlings, T.C., Guerin, P.M., Connetable, S., Kuhlemeier, C.,  
520 2005. The composition and timing of flower odour emission by wild *Petunia axillaris*  
521 coincide with the antennal perception and nocturnal activity of the pollinator *Manduca*  
522 *sexta*. *Planta*, 222: 141-150.

523 Hoballah, M.E., Gübitz, T., Stuurman, J., Broger, L., Barone, M., Mandel, T., Dell'Olivo A.,  
524 Arnold M., Kuhlemeier, C., 2007. Single gene-mediated shift in pollinator attraction in  
525 *Petunia*. *The Plant Cell*, 19: 779-790.

526 Jacquemyn, H., Brys, R., Adriaens, D., Honnay, O. & Roldán-Ruiz, I., 2009a. Effects of  
527 population size and forest management on genetic diversity and structure of the tuberous  
528 orchid *Orchis mascula*. *Cons. Genet.* 10: 161–168.

529 Jacquemyn, H., Brys, R., Honnay O., Hutchings, M.J., 2009b. Biological Flora of the British  
530 Isles: *Orchis mascula* (L.) L. *Journal of Ecol.* 97: 360–377

531 Jersáková, J., Johnson, S.D., Kindlmann, P., 2006. Mechanisms and evolution of deceptive  
532 pollination in orchids. *Biol. Rev.*, 81: 219–235.

533 Joffard, N., Massol, F., Grenié, M., Montgelard, C., Schatz, B., 2019. Effect of pollination  
534 strategy, phylogeny and distribution on pollination niches of Euro-Mediterranean orchids.  
535 *J. Ecol.*, doi: 10.1111/1365-2745.13013

536 Kessler, A., Halitschke, R., 2009. Testing the potential for conflicting selection on floral  
537 chemical traits by pollinators and herbivores: predictions and case study. *Funct. Ecol.*, 23:  
538 901-912.

539 Kessler, D., Diezel, C., Baldwin, I.T., 2010. Changing pollinators as a means of escaping  
540 herbivores. *Curr. Biol.*, 20: 237-242.

541 Kessler, D., Diezel, C., Clark, D.G., Colquhoun, T.A., Baldwin, I.T., 2013. Petunia flowers  
542 solve the defence/apparency dilemma of pollinator attraction by deploying complex floral  
543 blends. *Ecol. Lett.*, 16: 299–306.

544 Kindlmann, P., Jersáková, J., 2006. Effect of floral display on reproductive success in  
545 terrestrial orchids. *Folia Geobot.*, 41: 47–60.

546 Knudsen, J.T., Eriksson, R., Gershenzon, J., Stahl, B., 2006. Diversity and distribution of  
547 floral scent. *Bot. Rev.* 72: 1-120.

548 Kolosova, N., Gorenstein, N., Kish, C.M., Dudareva, N., 2001. Regulation of circadian  
549 methyl benzoate emission in diurnally and nocturnally emitting plants. *The Plant Cell*,  
550 13: 2333-2347.

551 Lawson, D.A., Chittka, L., Whitney, H.M., Rands, S.A., 2018. Bumblebees distinguish floral  
552 scent patterns, and can transfer these to corresponding visual patterns. *Proc. Roy. Soc. B:*  
553 *Biol. Sci.*, 285; 20180661.

554 Li ZG, Lee MR, Shen DL. 2006. Analysis of volatile compounds emitted from fresh *Syringa*  
555 *oblate* flowers in different florescence by headspace solid-phase microextraction-gas  
556 chromatography-mass spectrometry. *Anal. Chim. Acta*, 276: 43-49.

557 Lucas-Barbosa, D., van Loon, J.J., Dicke, M., 2011. The effects of herbivore-induced plant  
558 volatiles on interactions between plants and flower-visiting insects. *Phytochem.*, 72:  
559 1647-1654.

560 Majetic, C.J., Raguso, R.A., Tonsor, S.J., Ashman, T.-L., 2007. Flower color-flower scent  
561 associations in polymorphic *Hesperis matronalis* (Brassicaceae). *Phytochem.*, 68: 865-  
562 874.

563 Majetic, C.J., Raguso, R.A., Ashman, T.L., 2008. The impact of biochemistry vs. population  
564 membership on floral scent profiles in colour polymorphic *Hesperis matronalis*. *Ann.*  
565 *Bot.*, 102: 911-922.

566 Majetic, C.J., Raguso, R.A., Ashman, T.L., 2009. Sources of floral scent variation: can  
567 environment define floral scent phenotype? *Plant Signal. Behav.*, 4: 129-131.

568 Moya, S.J.D., Ackerman, J.D., 1993. Variation in the floral fragrance of *Epidendrum ciliare*  
569 (Orchidaceae). *Nord. J. Bot.*, 13: 41-47.

570 Muhlemann, J.K., Waelti, M.O., Widmer, A., Schiestl, F.P., 2006. Postpollination changes in  
571 floral odor in *Silene latifolia*: Adaptive mechanisms for seed-predator avoidance? *J.*  
572 *Chem. Ecol.*, 32: 1855–1860.

573 Negre, F., Kish, C.M., Boatright, J., Underwood, B., Shibuya, K., Wagner, C., Clark, D.G.,  
574 Dudareva, N., 2003. Regulation of methylbenzoate emission after pollination in  
575 snapdragon and petunia flowers. *Plant Cell*, 15: 2992-3006

576 Nilsson, L.A., 1978. Pollination ecology and adaptation in *Platanthera chlorantha*  
577 (Orchidaceae). *Bot. Not.*, 131: 35–51.

578 Nilsson LA. 1983. Anthecology of *Orchis mascula* (Orchidaceae). *Nord. J. Bot.*, 3: 157-179.

579 Odell, E., Raguso, R.A., Jones, K.N., 1999. Bumblebee foraging responses to variation in  
580 floral scent and color in snapdragons (*Anthirrhinum*: Scrophulariaceae). *Am. Midl. Nat.*,  
581 142: 257-265.

582 Okamoto, T., Kawakita, A., Kato, M., 2008. Floral adaptations to nocturnal moth pollination  
583 in *Diplomorpha* (Thymelaeaceae). *Plant Sp. Biol.*, 23: 192-201.

584 Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., Oksanen,  
585 M. J. (2013). Package 'vegan'. *Community ecology package*, version, 2: 1-295.

586 Olesen, J.M., Knudsen, J.T., 1994. Scent profiles of colour morphs of *Corydalis cava*  
587 (Fumariaceae) in relation to foraging behaviour of bumblebee queens (*Bombus*  
588 *terrestris*). *Biochem. Syst. Ecol.*, 22: 231-237.

589 Pellegrino, G., Bellusci, F., Musacchio, A. 2010. The effects of inflorescence size and flower  
590 position on female reproductive success in three deceptive orchids. *Bot. Stud.*, 51: 351-  
591 356.

592 Peter, C.I., Johnson, S.D., 2013. A pollinator shift explains floral divergence in an orchid  
593 species complex in South Africa. *Ann. Bot.*, 113: 277-288.

594 Prieto-Benítez, S., Dötterl, S., Giménez-Benavides, L., 2016. Circadian rhythm of a *Silene*  
595 species favours nocturnal pollination and constrains diurnal visitation. *Ann. Bot.*, 118:  
596 907–918.

597 Proffit, M., Schatz, B., Bessière, J.M., Chun Chen, C., Soler, C., Hossaert-McKey, M., 2008.  
598 Signalling receptivity: comparison of the emission of volatile compounds by figs of *Ficus*  
599 *hippida* before, during and after the phase of receptivity to pollinators. *Symbiosis*, 45: 15-  
600 24.

601 R Development Core Team, R. F. F. S. C., 2011. R: A language and environment for  
602 statistical computing.

603 Raguso, R.A., Light, D.M., Pickersky, E., 1996. Electroantennogram responses of *Hyles*  
604 *lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri*  
605 (Onagraceae) and other moth-pollinated flowers. *J. Chem. Ecol.*, 22: 1735-1766.

606 Raguso, R.A., Levin, R.A., Fooze, S.E., Holmberg, M.W., McDade, L.A., 2003. Fragrance  
607 chemistry, nocturnal rhythms and pollination “syndromes” in *Nicotiana*. *Phytochem.*, 63:  
608 265-284.

609 Raguso, R.A., 2008. Wake up and smell the roses: the ecology and evolution of floral scent.  
610 *Annu. Rev. Ecol. Evol. Syst.*, 39: 549-569.

611 Raguso, R., Weiss, M.R., 2015. Concerted changes in floral colour and scent, and the  
612 importance of spatio-temporal variation in floral volatiles. *J. Ind. Inst. Sci.*, 95: 69-92.

613 Salvagnin, U., Malnoy, M., Thöming, G., Tasin, M., Carlin, S., Martens, S., Vrhovsek, U.,  
614 Angeli, S., Anfora, G., 2018. Adjusting the scent ratio: using genetically modified *Vitis*  
615 *vinifera* plants to manipulate European grapevine moth behaviour. *Plant Biotechnol. J.*,  
616 16: 264-271.

617 Salzmann, C., Nardella, A.M., Cozzolino, S., Schiestl, F.P., 2007. Variability in floral scent in  
618 rewarding and deceptive orchids: the signature of pollinator-imposed selection? *Ann.*  
619 *Bot.* 100: 757-765.

620 Salzmann, C., Schiestl, F.P., 2007. Odour and colour polymorphism in the food-deceptive  
621 orchid *Dactylorhiza romana*. *Plant. Syst. Evol.*, 267: 37-45.

622 Schade, F., Legge, R.L., Thompson, J.E., 2001. Fragrance volatiles of developing and  
623 senescing carnation flowers. *Phytochem.*, 56: 703–710.

624 Schatz, B., Delle-Vedove, R., Dormont, L., 2013. Presence, distribution and pollination  
625 effects of white, pink and purple morphs in the orchid *Orchis mascula*. *Eur. J. Environ.*  
626 *Sci.*, 3: 119-128.

627 Schiestl, F., 2005. On the success of a swindle: pollination by deception in orchids.  
628 Naturwiss., 92: 255-264.

629 Schiestl, F.P., 2015. Ecology and evolution of floral volatile-mediated information transfer in  
630 plants. New Phytol., 206:5 71–77.

631 Schiestl, F.P., Ayasse, M., 2001. Post-pollination emission of a repellent compound in a  
632 sexually deceptive orchid: a new mechanism for maximising reproductive success?  
633 Oecologia, 126: 531-534.

634 Schlumpberger, B.O., Raguso, R.A., 2008. Geographic variation in floral scent of *Echinopsis*  
635 *ancistrophora* (Cactaceae); evidence for constraints on hawkmoth attraction. Oikos, 117:  
636 801-814.

637 Soler, C., Proffit, M., Bessiere, J.M., Hossaert-McKey, M., Schatz, B., 2012. Evidence for  
638 intersexual chemical mimicry in a dioecious plant. Ecol. Lett. 9: 978-985.

639 Steen, R., Norli, H.R., Thöming, G., 2019. Volatiles composition and timing of emissions in a  
640 moth-pollinated orchid in relation to hawkmoth (Lepidoptera: Sphingidae) activity.  
641 Arthropod Plant Interact., 1-12.

642 Steenhuisen, S.L., Jürgens, A., Johnson, S.D., 2013. Effects of volatile compounds emitted by  
643 *Protea* species (Proteaceae) on antennal electrophysiological responses and attraction of  
644 Cetoniine beetles. J. Chem. Ecol., 39: 438-446.

645 Steenhuisen, S.L., Raguso, R.A., Jürgens, A., Johnson, S.D., 2010. Variation in scent  
646 emission among floral parts and inflorescence developmental stages in beetle-pollinated  
647 *Protea* species (Proteaceae). South Afr. J. Bot., 76: 779–787.

648 Suetsugu, K., Naito, R. S., Fukushima, S., Kawakita, A., Kato, M., 2015. Pollination system  
649 and the effect of inflorescence size on fruit set in the deceptive orchid *Cephalanthera*  
650 *falcata*. J. Plant Res., 128: 585-594.

651 Suchet, C., Dormont, L., Schatz, B., Giurfa, M., Simon, V., Raynaud, C., Chave, J., 2011.  
652 Floral scent variation in two *Antirrhinum majus* subspecies influences the choice of naïve  
653 bumblebees. Behav. Ecol. Sociobiol., 65: 1015-1027.

654 Sun, M., Gross, K., Schiestl, F.P., 2014. Floral adaptation to local pollinator guilds in a  
655 terrestrial orchid. Ann. Bot., 113: 289-300.

656 Svensson, G.P., Hickman, M.O., Bartram, S., Boland, W., Pellmyr, O., Raguso, R.A., 2005.  
657 Chemistry and geographic variation of floral scent in *Yucca filamentosa* (Agavaceae).  
658 Am. J. Bot., 92: 1624-1631.

659 Tasin, M., Anfora, G., Ioriatti, C., Carlin, S., De Cristofaro, A., Schmidt, S., Bengtsson, M.,  
660 Versini, G., Witzgall, P., 2005. Antennal and behavioral responses of grapevine  
661 moth *Lobesia botrana* females to volatiles from grapevine. *J. Chem. Ecol.*, 31: 77-87.

662 Theis, N., Raguso, R.A., 2005. The effect of pollination on floral fragrance in thistles. *J.*  
663 *Chem. Ecol.*, 31: 2581-2600.

664 Theis, N., Lerdau, M., Raguso, R.A., 2007. The challenge of attracting pollinators while  
665 evading floral herbivores: patterns of fragrance emission in *Cirsium arvense* and *Cirsium*  
666 *repandum* (Asteraceae). *Int. J. Plant Sci.*, 168: 587–601.

667 Tollsten, L., Bergström, J., 1989. Variation and post-pollination changes in floral odors  
668 released by *Platanthera bifolia* (Orchidaceae). *Nord. J. Bot.*, 9: 359-362.

669 Turlings, T.C., Erb, M., 2018. Tritrophic interactions mediated by herbivore-induced plant  
670 volatiles: mechanisms, ecological relevance, and application potential. *Annu. Rev.*  
671 *Entomol.*, 63: 433-452.

672 Vittecoq, M., Djieto-Lordon, C., Buatois, B., Dormont, L., McKey, D., Blatrix, R., 2011. The  
673 evolution of communication in two ant-plant mutualisms. *Evol. Biol.*, 38: 360-369.  
674 doi:10.1007/s11692-011-9125-8

675 Wang, H., Zheng, P., Aoki, D., Miyake, T., Yagami, S., Matsushita, Y., Fukushima, K.,  
676 Nakagawa, M., 2018. Sexual and temporal variations in floral scent in the subdioecious  
677 shrub *Eurya japonica* Thunb. *Ecol. Evol.* 8: 8266-8272.

678 Wright, G.A., Lutmerding, A., Dudareva, N., Smith, B.H., 2005. Intensity and the ratios of  
679 compounds in the scent of snapdragon flowers affect scent discrimination by honeybees  
680 (*Apis mellifera*). *J. Comp. Physiol. A*, 191: 105–114.

681 Zucker, A., Tzfira, T., Ben-Meir, H., Ovadis, M., Shklarman, E., Itzhaki, H., Forkmann, G.,  
682 Martens, S., Neta-Sharir, I., Weiss, D., Vainstein, A., 2002. Modification of flower colour  
683 and fragrance by antisense suppression of the flavonone 3-hydroxylase gene. *Mol.*  
684 *Breed.*, 9: 33–41.

685

686

687 Table 1. Floral volatiles emitted by *O. mascula* inflorescences: list of volatile compounds, and  
688 quantitative changes under the influence of 5 sources of variation. RT: retention time; O:  
689 occurrence (n=162 samples); Mean: mean composition of floral volatiles, values are  
690 expressed as a percentage relative to total volatile compounds (mean composition for 20  
691 individuals sampled at mid-day, before pollination, without herbivore attack, in open  
692 grassland site, with mean size inflorescences, and no leaf spots). SE: standard error. For each  
693 factor, increased ( $\nearrow$ ) or decreased ( $\searrow$ ) of the relative amount of the compounds that contribute  
694 the most to the dissimilarity observed between the groups are presented (one-way SIMPER,  
695 testing factor day). For the columns “Flower age” and “Circadian rhythm”, the two arrows  
696 correspond to the two sampled periods (e.g. for Circadian rhythm, first arrow refers to 9.00 vs  
697 13.30, and second arrow to 13.30 vs 18.00).  
698  
699

	RT	O	Mean	SE	Factor of variation					
					Flower age	Circadian rhythm	Pollination	Herbivory	Habitat open vs wood	
<b>Fatty acid derivatives</b>										
3-hexen-2-one	4.42	34	1.78	0.40						
(Z)-3-hexenol	5.71	3	0.10	0.07						
6-methyl-heptanol-1	9.21	8	0.41	0.17						
decane	9.40	63	0.86	0.16						
3-hexenyl-acetate	9.60	58	3.88	0.62	$\searrow$				$\searrow$	
6-methyl-heptanol-2	10.41	12	0.54	0.21						
5-methyl-heptanol	10.71	4	0.20	0.11						
octatrienal	12.10	30	0.11	0.02						
6-methyl-heptanol-3	12.90	23	0.39	0.12						
methyl-octanoate	13.10	4	0.08	0.05						
hexenyl-butyrate	15.09	9	0.08	0.03						
nonan-2-one	15.90	5	0.03	0.02						
undecanal	18.37	35	0.25	0.05						
methyl-caprate	18.70	4	0.85	0.43						
tetradecanal	21.07	4	0.02	0.01						
butyl-octanol	23.30	31	0.16	0.03						
methyl-laurate	23.80	4	0.15	0.08						
butyl-caprate	26.50	33	0.41	0.09						
nonadecane	30.12	4	0.01	0.01						
docosane	33.19	5	0.05	0.02						
isopropyl-palmitate	33.90	10	0.08	0.05						
<b>Benzenoids</b>										
styrene	6.50	13	0.14	0.05						
acetophenone	11.49	13	0.47	0.17						
ethylbenzaldehyde	14.31	4	0.02	0.01						
cinnamaldehyde	14.96	6	0.02	0.01						
toluene-3-5-dimethoxy	17.23	3	0.06	0.04						
ethyl-2-benzofurane	17.70	4	0.02	0.01						
<b>Terpenoids</b>										
$\alpha$ -thujene	7.38	23	0.11	0.04						
$\alpha$ -pinene	7.60	159	6.57	0.46			$\nearrow$			
camphene	8.03	8	0.18	0.07						
sabinene	8.67	147	3.42	0.22			$\nearrow$	$\nearrow$		
$\beta$ -pinene	8.81	132	1.15	0.08			$\nearrow$	$\nearrow$		$\nearrow$
6-methyl-5-hepten-2-one	9.02	120	5.66	0.56	$\nearrow$	$\nearrow$	$\nearrow$	$\nearrow$		$\nearrow$
myrcene	9.15	106	6.26	0.70	$\searrow$	$\searrow$	$\nearrow$	$\nearrow$		$\nearrow$
3-thujene	9.62	2	0.01	0.01			$\nearrow$	$\nearrow$		$\nearrow$
$\alpha$ -terpinene	9.90	10	0.02	0.01						
p-cymene	10.20	58	0.31	0.05						
limonene	10.33	159	25.5	1.72	$\searrow$	$\searrow$	$\searrow$	$\searrow$		$\searrow$
phellandrene	10.34	4	0.19	0.10						
eucalyptol	10.40	111	4.18	0.52	$\nearrow$		$\nearrow$	$\nearrow$		$\nearrow$

(Z)-ocimene	10.50	47	0.76	0.11		↘	↗	↗
(E)-ocimene	10.80	70	8.80	1.18		↘	↗	↗
3-carene	11.13	19	0.02	0.01				
γ-terpinene	11.20	26	0.09	0.02				
sabinene-hydrate	11.57	104	1.76	0.16		↗	↘	↘
isopulegol	11.64	4	0.01	0.01				
3-caren-4-ol	11.84	7	0.01	0.01				
linalool-oxide	12.05	140	5.49	0.51				↘
2-pinen-10-ol	12.13	2	0.01	0.01	↗	↗		↘
linalool	12.44	135	7.66	0.68		↗	↘	↘
trans-p-mentha-2-8-dienol	13.08	5	0.01	0.01			↘	
allo-ocimene	13.25	43	0.65	0.10			↘	
limonene-oxide-trans	13.50	8	0.04	0.01				
pinocarveol-1	13.68	21	0.24	0.05				
cis-verbenol	13.72	2	0.03	0.03				
limonene-oxide-cis	14.08	6	0.01	0.01				
pinocarveol-2	14.19	7	0.01	0.01				
verbenone	14.20	22	0.22	0.05				
citronellol	14.48	35	0.18	0.04				
epoxy-linalool	14.66	17	0.05	0.01				
α-terpineol	15.25	64	0.84	0.13				
safranal	15.61	27	0.15	0.04				
exo-2-hydroxy-cineole	16.14	4	0.01	0.01				
carvone	16.62	13	0.04	0.01				
bergamiol	16.71	3	0.03	0.02				
ethyl-linalool	17.07	8	0.08	0.03				
8-hydroxy-linalool	17.52	8	0.03	0.01				
3-caren-4-ol	19.26	3	0.02	0.01				
α-terpineol-acetate	19.40	22	0.17	0.04				
β-bourbonene	20.41	2	0.01	0.01				
aromadendrene	21.00	5	0.03	0.01				
β-caryophyllene	21.39	20	0.75	0.27				
caryophyllene-2	21.60	6	0.05	0.03				
α-bergamotene	21.61	24	0.33	0.08				
geranylacetone	22.02	23	0.25	0.06				
β-farnesene	22.10	11	0.47	0.16				
germacrene-D	22.72	2	0.01	0.01				
α-curcumene	22.85	20	0.14	0.04				
Z-E-α-farnesene	23.04	6	0.02	0.01				
α-farnesene-2	23.40	13	0.12	0.04				
α-longipinene	23.50	24	0.42	0.09				
α-patchoulene-1	24.52	2	0.01	0.01				↘
caryophyllene-oxide-1	24.74	4	0.01	0.01				
longipinocarvone	25.16	3	0.02	0.01				
caryophyllene-oxide-2	25.36	13	0.20	0.11				
longipinocarvone-2	25.41	8	0.07	0.03				
α-patchoulene-2	25.61	6	0.02	0.01				
caryophyllene-oxide-3	25.71	6	0.02	0.01				
aromadendrene-oxide	26.04	3	0.01	0.01				
α-patchoulene-3	26.13	4	0.01	0.01				
caryophyllene-oxide-4	26.60	3	0.03	0.03				
E-E-farnesol	30.74	2	0.01	0.01				

700

701

702 Table 2. Results of the statistical test performed on volatile compound proportions emitted by  
703 *Orchis mascula*. The significance of each factor was tested independently using a  
704 permutational analysis of variance (PERMANOVA), and the homogeneity of variance groups  
705 was tested using a multivariate Levene's test. Before analysis, data were transformed using  
706 squared root and Wisconsin double standardization, followed by a matrix of pairwise  
707 comparisons using Bray-Curtis dissimilarity indices.  
708

Factor of variation	PERMANOVA				homogeneity variance	
	DF	(F)	r <sup>2</sup>	p	(F)	p
Flower age	2; 47	4.696	0.167	0.001	5.472	0.007
Circadian	2; 52	2.393	0.084	0.001	3.118	0.053
Herbivory	1; 28	3.006	0.097	0.001	0.698	0.411
Pollination	1; 28	3.806	0.120	0.001	0.958	0.336
Habitat	1; 38	3.386	0.082	0.001	0.008	0.931
Inflorescence	3;65	1.111	0.049	0.270	1.943	0.132
Spots leaves	1; 68	1.074	0.016	0.359	0.329	0.568

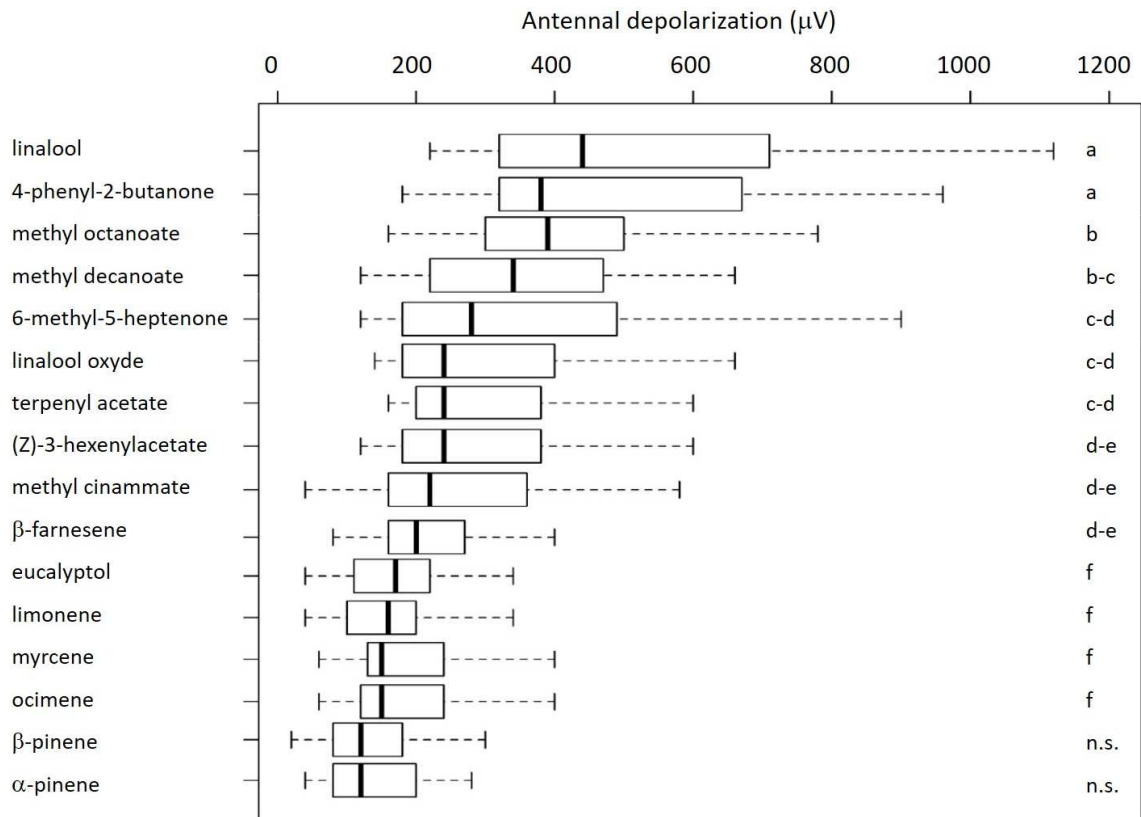
709  
710

711 Fig. 1: Non-metric multi-dimensional scaling of the relative proportions of *O. mascula* floral  
712 volatiles under the influence of different factors of scent variation.

713

714 Fig. 2: Average EAG responses of female *Bombus terrestris* antennae (n=31 individuals) to  
715 synthetic compounds identified from *Orchis mascula* floral volatiles. Each synthetic  
716 compound was individually tested at different dilutions, the figure shows results with dilution  
717 1% v/v in dichloromethane. Bars followed by the same letter were not significantly different  
718 (Wilcoxon test). n.s.: EAG response was not significantly different to the depolarization  
719 recorded with a control stimulus (dichloromethane extract).

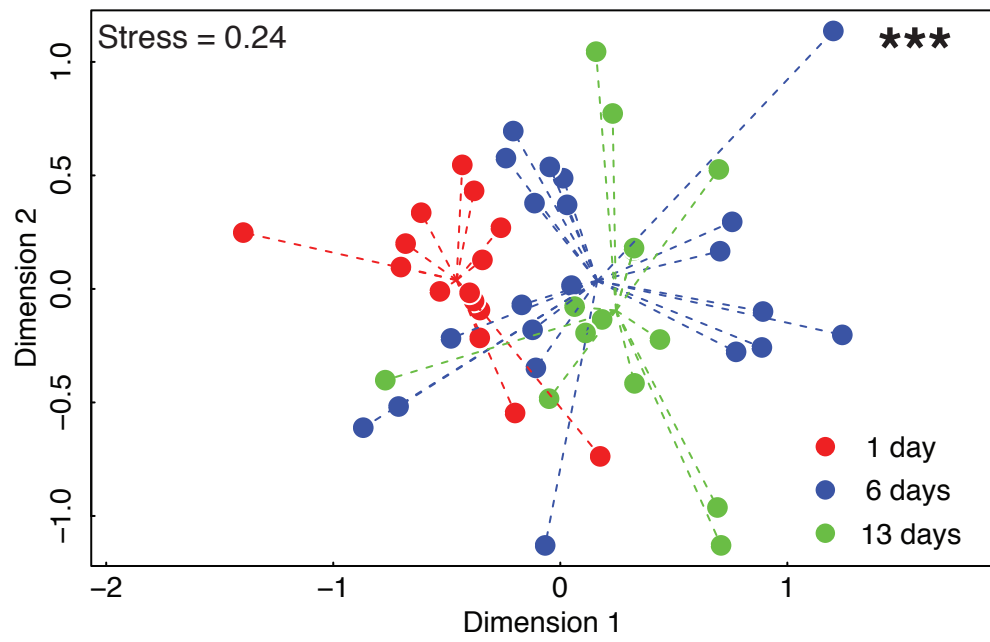
720



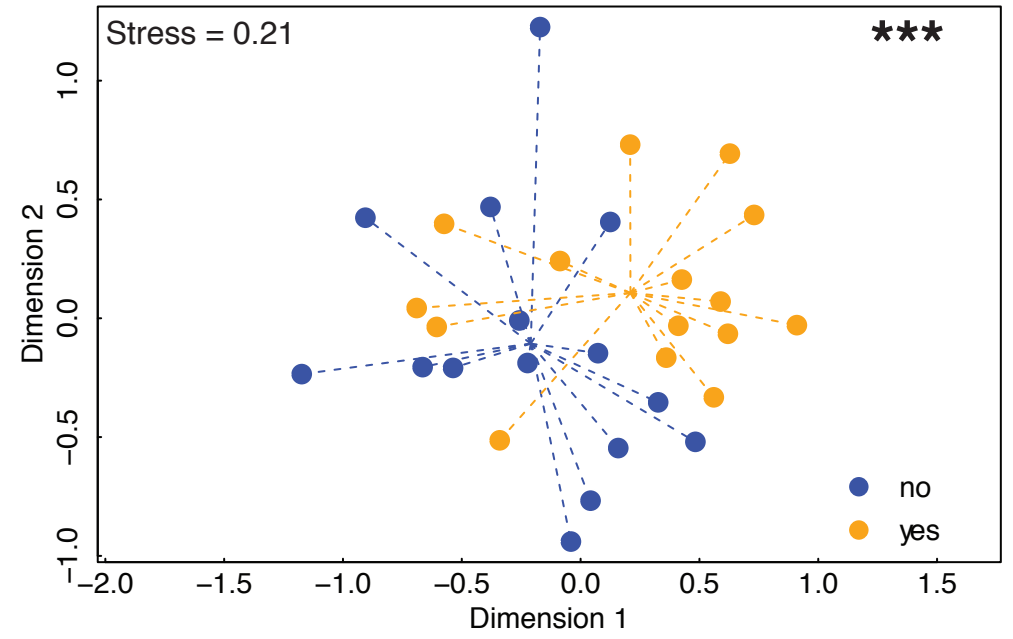
721

722

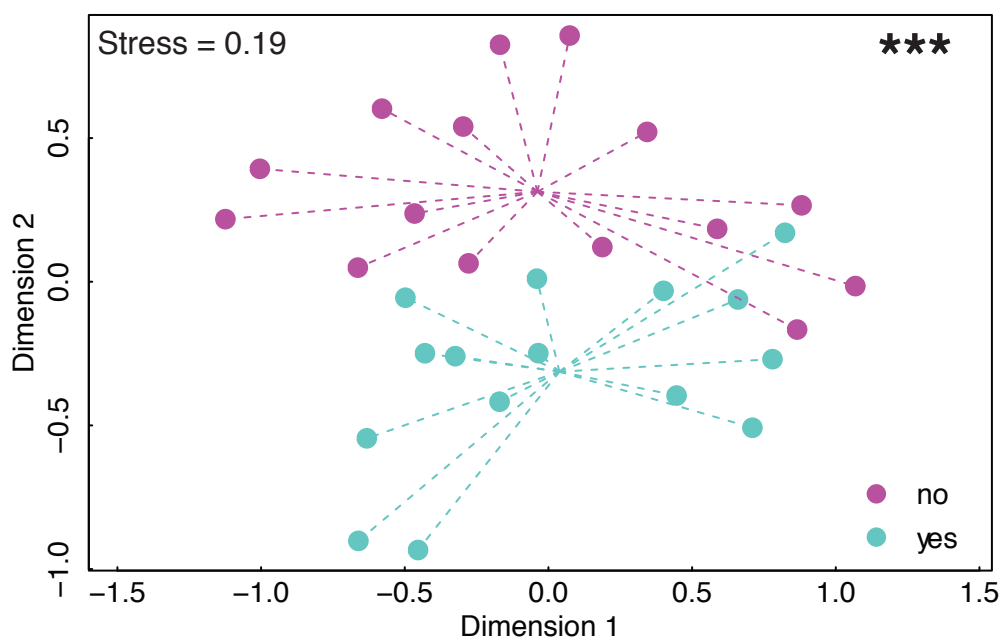
## Flower age



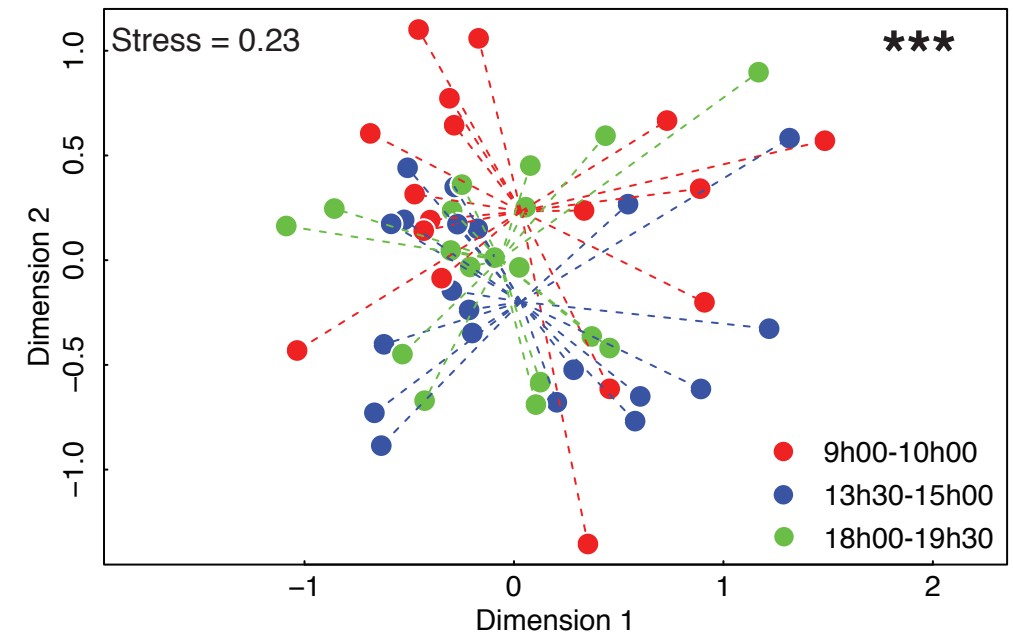
## Pollination



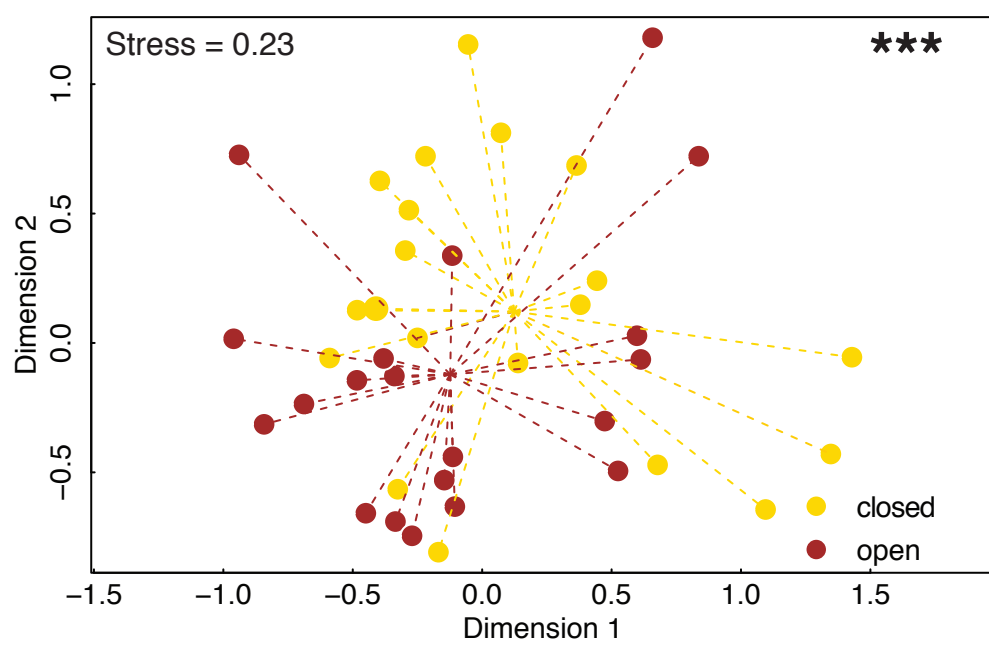
## Herbivory



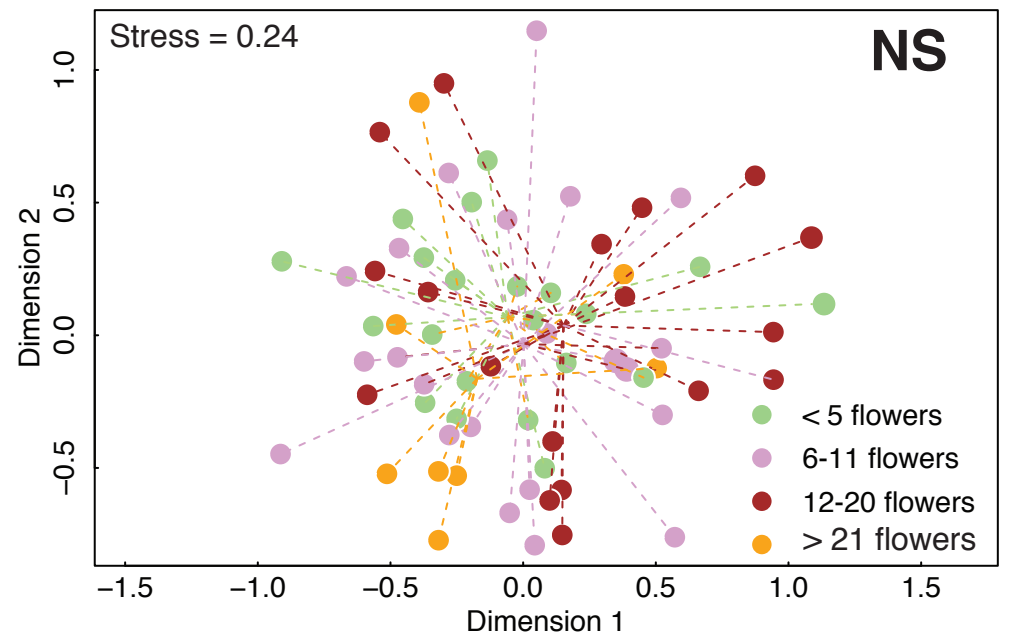
## Circadian rhythm



## Habitat



## Inflorescences



## Spot leaves

