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

2 ***mascula***

3

4

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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; Salzmann 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 μm
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⁻¹), and programmed 2 min isothermal at 50 °C, then 50 °C to 220 °C at 4 °C
195 min⁻¹. 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⁻¹ NaCl, 0.4 gl⁻¹
212 KCl, 0.4 gl⁻¹ CaCl₂) 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⁻¹, 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, α -pinene, 6-methyl-
224 5-hepten-2-one, linalool-oxide, eucalyptol, myrcene, methyl-decanoate, β -farnesene, 4-
225 phenyl-2-butanone, methyl-octanoate, terpinyl-acetate, β -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 μ 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⁻¹ 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 μ 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%), α -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, β -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 β -
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 α -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. α -pinene and β -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 Salzman 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

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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**

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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	
Fatty acid derivatives										
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						
Benzenoids										
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						
Terpenoids										
α -thujene	7.38	23	0.11	0.04						
α -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		
β -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
α -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 ²	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

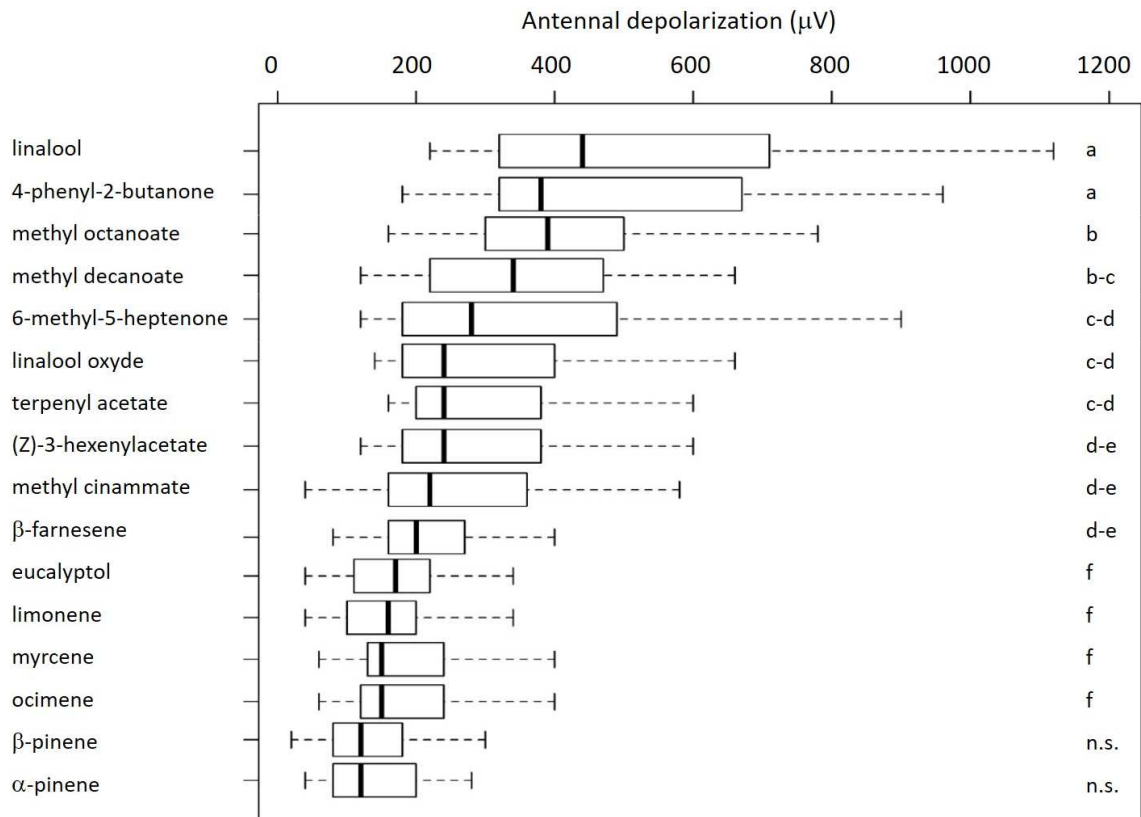
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711 Fig. 1: Non-metric multi-dimensional scaling of the relative proportions of *O. mascula* floral
712 volatiles under the influence of different factors of scent variation.

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).

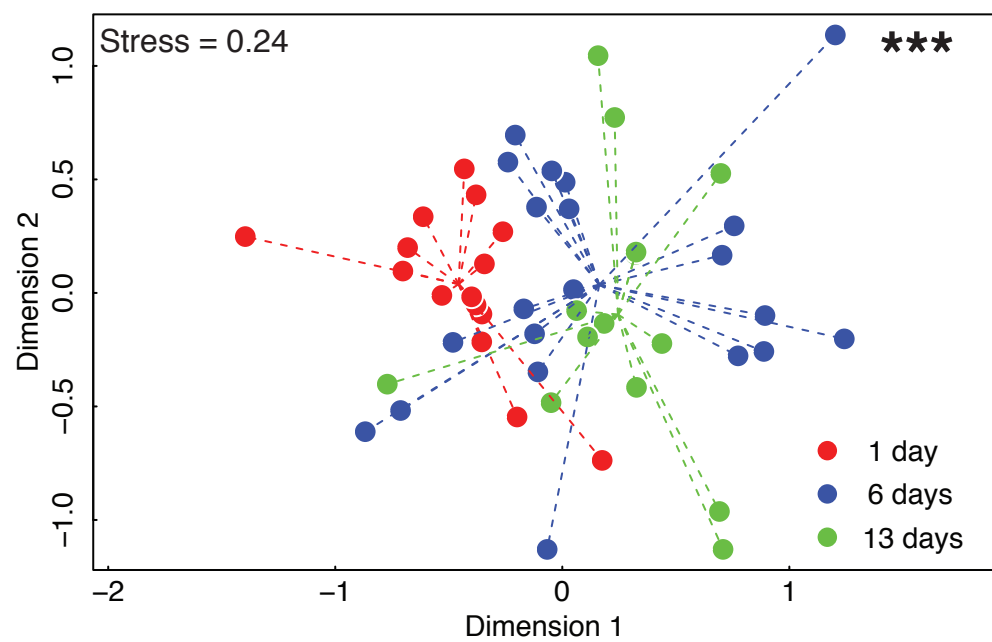
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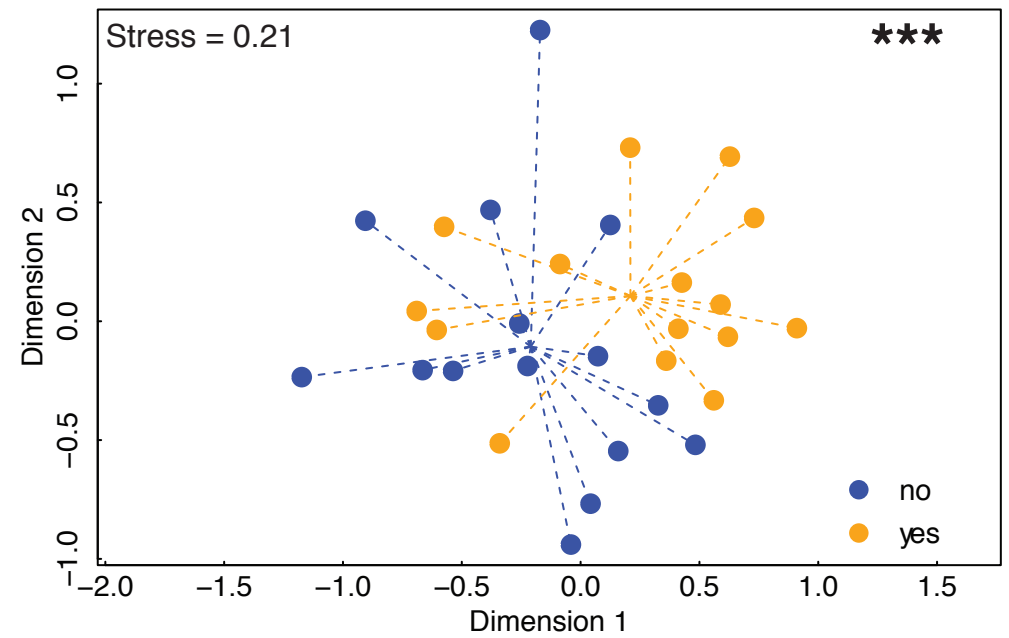
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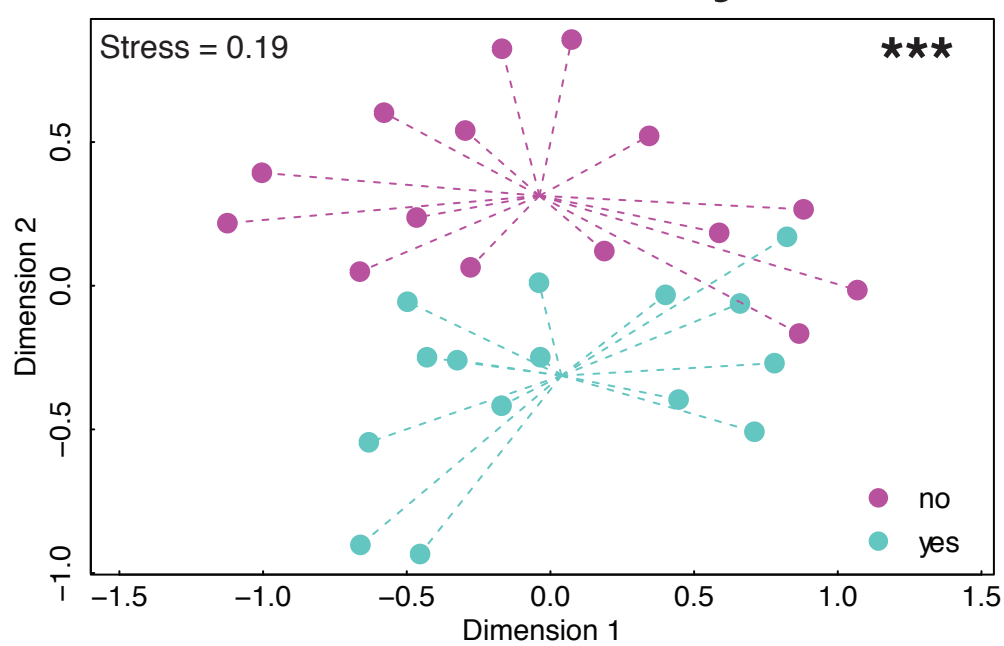
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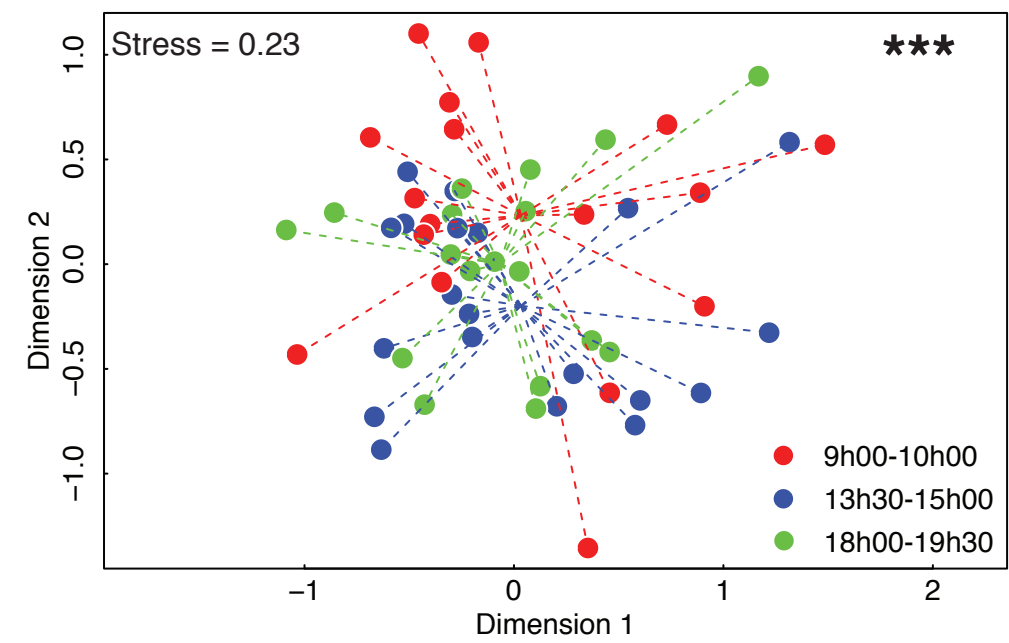
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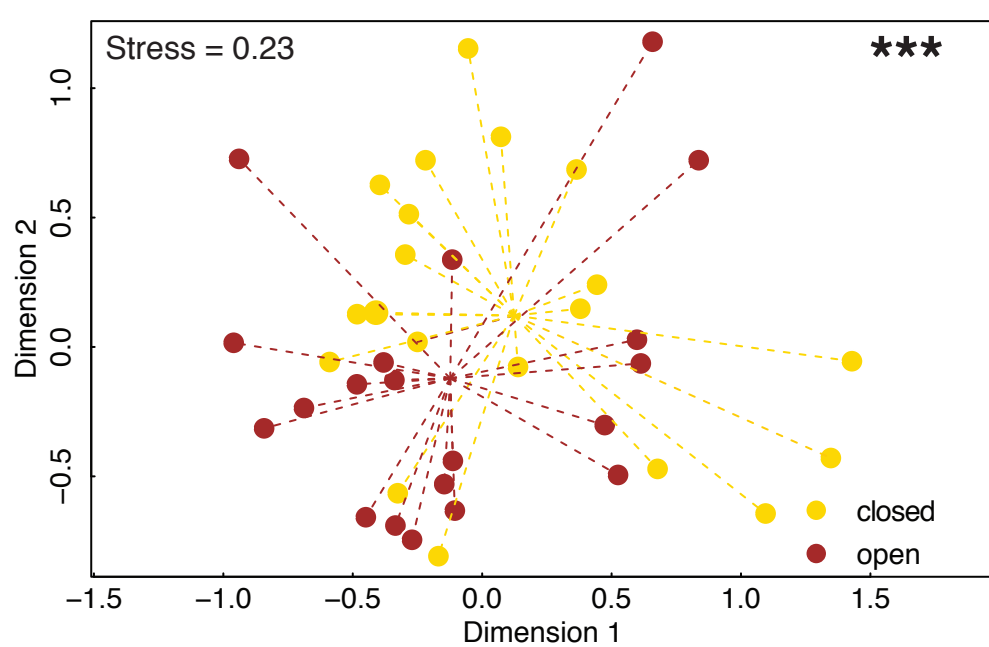
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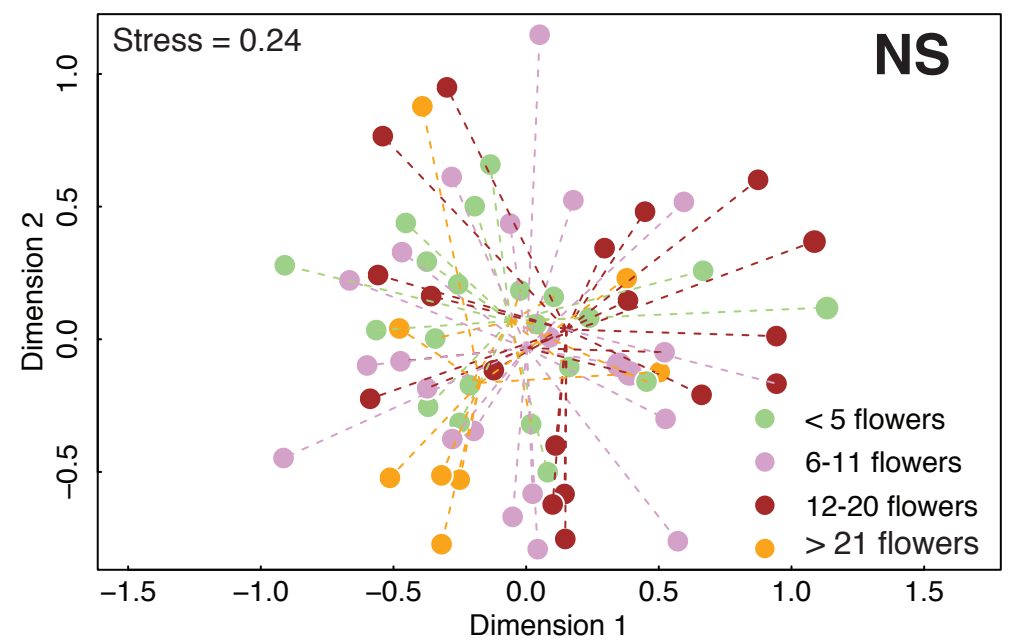
Circadian rhythm



Habitat



Inflorescences



Spot leaves

