

**Title: Ozone degrades floral scent and reduces pollinator attraction to flowers**

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## 1 SUMMARY

- 2 • In this work we analyzed the degradation of floral scent volatiles from *Brassica nigra*  
3 by reaction with ozone along a distance gradient and the consequences for pollinator  
4 attraction.
- 5 • For this purpose we used a reaction system comprising three reaction tubes where we  
6 conducted measurements of floral volatiles by PTR-TOF-MS and GC-MS. We also  
7 tested the effects of floral scent degradation on the responses of the generalist pollinator  
8 *Bombus terrestris*.
- 9 • The chemical analyses revealed that supplementing air with ozone led to an increasing  
10 reduction in the concentrations of floral volatiles in air with distance from the volatile  
11 source. The results reveal different reactivities with ozone for different floral scent  
12 constituents, which emphasizes that ozone exposure not only degrades floral scents, but  
13 also changes the ratios of compounds in a scent blend. Behavioral tests revealed that  
14 floral scent was reduced in its attractiveness to pollinators after it had been exposed to  
15 120 ppb O<sub>3</sub> over a 4.5 m distance.
- 16 • The combined results of chemical analyses and behavioral responses of pollinators  
17 strongly suggest that high ozone concentrations have significant negative impacts on  
18 pollination by reducing the distance over which floral olfactory signals can be detected  
19 by pollinators.

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

22

23

## 24 **INTRODUCTION**

25 Volatile organic compounds (VOCs) mediate several ecological interactions between  
26 plants and other organisms (Dudareva *et al.*, 2006; Dicke & Baldwin, 2010). One of the  
27 ecological interactions mediated by VOCs is the communication between  
28 entomophilous plants and their respective pollinators (Farré-Armengol *et al.*, 2013). The  
29 establishment of such an interaction relies on plants producing chemical scent cues that  
30 can be identified by pollinators and facilitate communication over scales ranging from  
31 short to long-distance. These chemical cues can provide diverse information to  
32 pollinators, such as the species to which they belong, the availability and quality of  
33 rewards (Howell & Alarcón, 2007; Wright *et al.*, 2009), flower ontogeny (Mactavish &  
34 Menary, 1997; Goodrich *et al.*, 2006) and pollination state (Negre *et al.*, 2003). Floral  
35 scent cues also serve pollinators in their quest to locate the emitting source (flower) via  
36 scent trails that occur with concentration gradients (Cardé & Willis, 2008; Riffell *et al.*,  
37 2008).

38 Ozone is a powerful oxidizing agent and a common atmospheric pollutant in the  
39 lower atmosphere that may react with and disturb these floral scents. Tropospheric  
40 ozone concentration has significantly increased since pre-industrial era times due to  
41 anthropogenic activity (IPCC, 2001, 2007, 2013), and it is predicted to increase more in  
42 the next decades, enhanced by global warming and changes in land cover (Val Martin *et al.*,  
43 2014). Ozone has direct harmful effects on many living organisms including plants  
44 and animals (Mcgrath *et al.*, 2001; Kampa & Castanas, 2008; Díaz-de-Quijano *et al.*,  
45 2012). Ozone can have significant negative impacts on plant reproductive success via its

46 negative impacts on plant tissues and plant physiology (Bergweiler & Manning, 1999;  
47 Black *et al.*, 2007). Furthermore, many recent studies have reported that ozone and  
48 other common oxidative pollutants, such as hydroxyl and nitrate radicals, affect the  
49 emissions of VOCs from plants and the interactions they mediate (Pinto *et al.*, 2007a,  
50 2010; McFrederick *et al.*, 2009; Blande *et al.*, 2010, 2011; Fuentes *et al.*, 2013).  
51 Tropospheric ozone can affect plant emissions and their effectiveness in two ways: first,  
52 by affecting plant physiology and inducing changes in the emission profiles  
53 (Andermann *et al.*, 1999; Peñuelas & Llusia, 1999; Holopainen & Gershenzon, 2010),  
54 and second, by mixing and reacting with the emitted compounds once they are released  
55 (Holopainen & Blande, 2013; Blande *et al.*, 2014).

56         The oxidative degradation of the VOCs emitted by flowers may reduce their  
57 concentration in an odor plume, decreasing the distances they can travel before reaching  
58 concentrations that are not detected by foraging pollinators (McFrederick *et al.*, 2008).  
59 Moreover, the reactivity of the individual VOCs in a blend differs both with the  
60 properties of the chemical and the properties of the oxidizing agent. Therefore, VOCs in  
61 a chemical blend may be degraded at different rates in ozone polluted (Atkinson & Arey,  
62 2003) or in diesel fume (NO and NO<sub>2</sub>) polluted environments (Girling *et al.*, 2013),  
63 leading to changes in the original ratios of VOC in the floral scent (McFrederick *et al.*,  
64 2009). The oxidative reactions of ozone with plant-emitted VOCs lead to the formation  
65 of new organic compounds that can be volatile and persistent in the altered volatile  
66 blend (Pinto *et al.*, 2010). These *de novo* produced compounds are not part of the  
67 original scent of the species, and may induce confusion in the signal receivers, in this  
68 case pollinators, if they are able to detect its presence. All processes involving the  
69 reaction of ozone with VOCs may reduce the intensity of floral scent and provide  
70 significant additional variability to flower olfactory signals once they have been

71 released, potentially with negative effects on the reliability of floral scent as an  
72 attractant.

73 The objective of this work was to analyze the effects of exposure to different ozone  
74 concentrations on the floral scent of *Brassica nigra*, while testing the effects of induced  
75 changes on the attraction of the generalist pollinator *Bombus terrestris*. The sensory  
76 abilities of bumblebees and their learning and memory capabilities are well known,  
77 which makes them one of the most suitable models for conducting behavioral studies  
78 (Chittka & Raine, 2006; Riveros & Gronenberg, 2009). *Bombus terrestris* is one of the  
79 most abundant and widespread bumblebee species in the West Palearctic and has a very  
80 relevant role as a pollinator in wild and cultivated plant communities (Rasmont *et al.*,  
81 2008). The flower foraging preferences of *B. terrestris* display a large degree of  
82 generalism, which makes them a good pollination vector for a wide range of  
83 entomophilous plant species (Fontaine *et al.*, 2008). We expected floral scent to suffer  
84 quantitative and qualitative changes when exposed to ozone-enriched ambient air. We  
85 hypothesized (1) that floral scents would experience a greater degree of degradation  
86 with increasing distance from the scent source under higher ozone concentrations. We  
87 also hypothesized (2) that floral VOC mixtures might experience qualitative changes  
88 due to variation in the relative ratios of the existing compounds due to differences in  
89 their reactivity times with ozone, and also due to the formation of new compounds  
90 resulting from oxidative reactions of VOCs with ozone. With respect to flower-  
91 pollinator communication, we hypothesized (3) that pollinators would be more attracted  
92 to floral scent when it had not been exposed to ozone, than after being exposed to  
93 ozone-enriched ambient air over the longer distances tested.

## 94 **MATERIALS AND METHODS**

95 ***Brassica nigra* plants and flower collection**

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

108

109 **Chemical measurements**

110 **Experimental design**

111 We exposed the flower VOC emissions to 3 different ozone concentrations, 0, 80 and  
112 120 ppb. For each ozone concentration tested, we measured VOC concentrations with a  
113 PTR-TOF-MS at 4 distances from the scent source within the reaction system (0 m, 1.5  
114 m, 3 m and 4.5 m) (Figure 1). We repeated the measurements of VOC concentrations  
115 with eight different batches of flowers (weighing 1–2.5 g dry weight). We also sampled  
116 floral volatiles with adsorbent-filled tubes for each concentration and distance (n = 2-4)  
117 and analyzed them by GC-MS. We used STATISTICA version 8.0. (StatSoft, Inc.,  
118 Tulsa, USA, 2007), to conduct general linear models testing the effect of ozone

119 concentration and distance on floral VOC concentrations and also on the relative ratios  
120 of terpenes.

121

## 122 **Ozone reaction system**

123 We used an ozone reaction system comprising three glass tubes of 1.5 m length and 5.5  
124 cm inner diameter that were connected in sequence with metal tubes of 4 mm inner  
125 diameter. The system allowed the collection of air at 4 different distances from the  
126 emission source (Figure 1). We used an activated carbon filter to clean the air entering  
127 the system of any VOCs. The cut flowers were put into a sealed glass jar where an  
128 incoming clean air flow of  $900 \text{ mL min}^{-1}$  was regulated with a mass flow controller  
129 (Alicat Scientific, AZ, USA). The clean air was mixed with floral volatile emissions  
130 inside the jar and was directed to the reaction system through Teflon tubing. Just before  
131 the entrance to the first reaction chamber, a tube connected to an ozone generator  
132 (Stable Ozone Generator, SOG-2; UVP, LLC-Upland, CA, USA) and carrying ozone  
133 enriched air at a mass flow controller regulated rate of  $50 \text{ mL min}^{-1}$  was joined to the  
134 tube carrying the floral volatile emissions. The first port from which air samples could  
135 be taken for chemical measurements and behavioral tests was situated just after the  
136 point that the two inlet flows mixed. The first port was named “distance 0”, after which  
137 the reaction system continued with three sequential reaction chambers, with further  
138 ports at the end of each chamber (distances 1, 2 and 3, at 1.5 m, 3 m and 4.5 m  
139 respectively) and an outlet at the end connected to an ozone scrubber. We used Teflon  
140 tubes of 4 mm inner diameter to connect the pump, the VOC filter, the ozone generator  
141 and the flower jar to the reaction system. We used an Ozone analyzer (Dasibi 1008-RS;

142 Dasibi Environmental Corp., Glendale, CA, USA) to calibrate and check the ozone  
143 concentrations achieved inside the reaction system.

144

#### 145 **PTR-TOF-MS measurements**

146 A high-resolution proton-transfer reaction time-of-flight mass spectrometer (PTR-TOF-  
147 MS 8000, Ionicon Analytik, Innsbruck, Austria) was used to monitor floral VOC  
148 concentrations. Sample air from the chamber was introduced into the PTR drift tube via  
149 a 1.5 m length (outside diameter 1/16 inch) of heated (60°C) PEEK tubing at a flow rate  
150 of 200 mL min<sup>-1</sup>. Hydronium ions (H<sub>3</sub>O<sup>+</sup>) were used as reagent ions to ionize organic  
151 compounds. The PTR-TOF-MS was operated under controlled conditions (2.3 mbar  
152 drift tube pressure, 600 V drift tube voltage and 60°C temperature). The raw PTR-TOF  
153 data were post-processed with the PTR-MS Viewer program (Ionicon Analytik).  
154 Concentrations were calculated by the program using a standard reaction rate constant  
155 of  $2 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1} \text{ molecule}^{-1}$ .

156

#### 157 **Volatile collection and GC-MS measurements**

158 We collected air from each of the sampling ports into adsorbent-filled tubes for a more  
159 detailed analysis of the floral terpene emissions by GC-MS. The tubes were filled with  
160 adsorbents Tenax® and Carbopack™ (150 mg each; Markes International, Llantrisant,  
161 RCT, UK). A sampling air flow of 200 mL min<sup>-1</sup> and sampling times of 30–40 min  
162 were used. The VOC samples were analysed by a GC-MS system (Agilent 7890A GC  
163 and 5975C VL MSD; New York, USA) with an approximate detection limit of 3 ng/mL.  
164 Trapped compounds were desorbed with an automated thermal desorber (TD-100;



165 Markes International Ltd, Llantrisant, UK) at 250°C for 10 min, cryofocused at -10°C  
166 and then transferred in a splitless mode to an HP-5 capillary column (50 m × 0.2 mm;  
167 film thickness 0.33 µm). Helium was used as a carrier gas. Oven temperature was held  
168 at 40°C for 1 min, then programmed to increase by 5°C min<sup>-1</sup> to 210°C, and then by  
169 20°C min<sup>-1</sup> to 250°C under a column flow of 1.2 mL min<sup>-1</sup>. The column effluent was  
170 ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning  
171 from 35-350 m/z with a scan rate of 5.38 scan/s.

172

### 173 **Testing the responses of pollinators**

174

#### 175 ***Bombus terrestris***

176 For the behavioral tests we used the bumblebee, *Bombus terrestris*, which was obtained  
177 as a group of three colonies each with a queen and providing an estimated 350-400  
178 individuals, including adult workers, pupae, larvae and eggs (TRIPOL, Koppert  
179 Biological Systems, Netherlands). The bumblebees were kept in two conjoined  
180 ventilated polycarbonate cages giving a total foraging area of 1.4 m × 1 m × 0.7 m. The  
181 box containing the bumblebee colonies was put in one cage and the other cage was used  
182 to provide *Brassica nigra* flowers and a 50% sucrose solution to feed the bumblebees.  
183 We regularly provided fresh *Brassica nigra* flowers to familiarize the bumblebees with  
184 the floral scent and associated reward. The colonies remained in healthy condition and  
185 provided adult individuals that were suitable for behavioral tests throughout the 1 month  
186 period of the behavioral study.

187

188 **Experimental design**

189 We conducted behavioral tests to assess the preferences of *B. terrestris* presented with  
190 the three following odour combinations:

- 191 1. “floral scent from distance 0 at 0 ppb O<sub>3</sub>” vs. “clean air” (n = 21).
- 192 2. “floral scent from distance 3 at 120 ppb O<sub>3</sub>” vs. “clean air” (n = 24).
- 193 3. “floral scent from distance 0 at 120 ppb O<sub>3</sub>” vs. “floral scent from distance 3 at  
194 120 ppb O<sub>3</sub>” (n = 21).

195 Floral scent sources were channeled from the port of the ozone exposure system  
196 corresponding with the distance and ozone treatment. The clean air comparison was  
197 first filtered and then passed through a glass jar with a pot of water to best match the  
198 humidity of the air exiting the reaction tubes. We conducted  $\chi^2$  tests to analyze the  
199 existence of pollinator preferences between compared air samples. We used paired t-  
200 tests to compare pollinator visitation between the artificial flowers of compared air  
201 samples.

202

203 **Behavioral chamber**

204 Behavioral tests were conducted in a cylindrical chamber made of transparent  
205 polycarbonate with a 1 m height and 1.5 m diameter (Figure 2). The lateral walls of the  
206 chamber were covered with light green paper to avoid interferences in bumblebee  
207 behavior due to visual interferences from outside the chamber. Two lamps were used as  
208 a light source and were positioned on the top of the behavioral chamber one on each  
209 side. The chamber had a 20 cm × 30 cm window at a central point in the side. Two  
210 metal tubes of approximately 1 m length and 4 mm inner diameter were inserted into the

211 cage entering from the top and positioned at opposite sides of the chamber. The metal  
212 tubes were connected to the two incoming air sources to be tested against each other  
213 inside the behavioral chamber. The metal tubes had some holes in the section, which  
214 released the odour sources close to artificial inflorescences that were placed in a metal  
215 support on the floor of the chamber. The artificial inflorescences consisted of yellow  
216 non-scented paper cut into the shape of petals and attached to a thin white Teflon tube  
217 with pins; the model resembled an inflorescence of *Brassica nigra*. Each inflorescence  
218 consisted of 8 flowers with position rotated around the tube. A third metal tube with the  
219 same dimensions was inserted in the center of the chamber. This tube had many holes  
220 all along its length oriented to all directions and was connected to a pump to draw air  
221 from the chamber (Figure 2).

222

### 223 **Behavioral tests**

224 Before starting the behavioral tests a series of checks and calibrations were conducted.  
225 First, the reaction system was turned on and outlet emissions were monitored by PTR-  
226 TOF-MS until a steady state was reached. After that we connected the two air sources  
227 that we wanted to test to the behavioral chamber. The pumps were turned on and the  
228 two incoming air flows were adjusted to  $500 \text{ mL min}^{-1}$  and the central outlet tube to  $1 \text{ L}$   
229  $\text{min}^{-1}$  (Figure 2). We then waited for another 30 minute period for the stabilization and  
230 homogenization of the air flows and VOC concentrations in the behavioral chamber  
231 system. For each test an individual bumblebee was collected from the colony in the dark  
232 and taken in a small pot to the adjacent lab where the behavioral chamber was housed.  
233 Each bumblebee was released from a central point of the chamber equidistant from the  
234 odour sources. At the start of the test the two lamps were turned on and the clock was

235 started when the bumblebee started to fly. Each bioassay was observed continually for  
236 10 minutes. The chamber was divided into two halves – one for each odour source –  
237 and the time spent in each half was recorded. When a bumblebee spent 315 seconds or  
238 more in one of the two halves, a choice for the respective odour source was assigned.  
239 However, when the times spent in each half differed in less than 30 seconds we  
240 determined that the test resulted in no choice. We also recorded the number of visits that  
241 the bees made to the artificial inflorescences. A visit was considered to have occurred  
242 when a flying bumblebee landed on one of the artificial inflorescences. Short flight  
243 movements between flowers within the same inflorescence were not considered to be  
244 different visits. If the bumblebees left the inflorescence, flew in the open chamber and  
245 landed again, we considered it a new visit. In addition, we transformed the data on  
246 pollinator visitation into a binary variable (0/1) for the statistical analyses. We assigned  
247 the value zero when no visits were conducted to artificial flowers during the test and we  
248 assigned the value one when pollinators conducted one or more visits. Once the test  
249 finished we released the bumblebees in a separate cage to avoid using the same  
250 individual for different test replicates on the same day, and we took a new bumblebee  
251 for the next trial.

252

## 253 **RESULTS**

### 254 **Effects of ozone on the chemistry of floral emissions**

255 Ozone concentration and distance from the floral scent source had a negative effect on  
256 the concentration of floral scent volatiles (Figure 3). Monoterpene (m/z 137.133),  
257 anisaldehyde (m/z 137.1562), and phenol (m/z 95.1194) concentrations showed very  
258 significant negative correlations with ozone concentration ( $P < 0.0001$ ), distance

259 ( $P<0.0001$ ) and the interaction between ozone concentration and distance ( $P<0.0001$ ).  
260 *p*-Cymene (m/z 135.1174) concentration also showed a very significant negative  
261 correlation with ozone concentration ( $P<0.0001$ ) and distance ( $P=0.013$ ). However,  
262 benzaldehyde (m/z 107.0497) concentration increased with ozone concentration ( $P=0.8$ )  
263 and distance ( $P=0.3$ ), although the effects were not found to be significant (Figure 4).

264 Under the highest ozone concentration tested, at the longest distance from the  
265 scent source (4.5 m), monoterpene concentration decreased by 26.4%, anisaldehyde  
266 decreased by 27%, phenol decreased by 29.5%, *p*-cymene decreased by 31% and  
267 benzaldehyde increased by 17%. These compound-specific responses lead to changes in  
268 the relative composition of floral VOC blends. A detailed analysis of the composition of  
269 floral terpene emissions by GC-MS showed gradual changes with distance when  
270 exposed to ozone, although changes were not found to be significant (Figure 5). When  
271 exposed to increasing ozone concentrations the monoterpenes  $\beta$ -myrcene,  $\beta$ -thujene,  
272 (*Z*)- $\beta$ -ocimene and  $\gamma$ -terpinene showed gradual relative increases with respect to other  
273 terpene compounds, while  $\alpha$ -pinene gradually decreased.

274

## 275 **Pollinator responses in behavioural tests**

276 Bumblebees showed a clear orientation bias toward “*floral scent from distance 0 at 0*  
277 *ppb O<sub>3</sub>*” over “*clean air*” ( $\chi^2$  test,  $P=0.01$ ) (Figure 6A). From a total of 21 tests, thirteen  
278 bumblebees spent more time in the half of the arena with “*floral scent from distance 0*  
279 *at 0 ppb O<sub>3</sub>*”, three spent more time in the half with “*clean air*”, and five individuals did  
280 not make a clear choice. Bumblebees showed no clear orientation bias when presented  
281 with “*floral scent from distance 3 at 120 ppb O<sub>3</sub>*” and “*clean air*” ( $\chi^2$  test,  $P=0.37$ )  
282 (Figure 6B). From a total of 22 tests, eight bumblebees spent more time in the half with

283 “floral scent from distance 3 at 120 ppb O<sub>3</sub>”, twelve of them spent more time in the half  
284 with “clean air”, and two individuals did not make a clear choice. Finally, bumblebees  
285 showed a marked orientation bias toward “floral scent from distance 0 at 120 ppb O<sub>3</sub>”  
286 over “floral scent from distance 3 at 120 ppb O<sub>3</sub>” ( $\chi^2$  test,  $P=0.005$ ) (Figure 6C). From a  
287 total of 21 tests, fifteen bumblebees spent more time in the half with “floral scent from  
288 distance 0 at 120 ppb O<sub>3</sub>”, three of them spent more time in the half with “floral scent  
289 from distance 3 at 120 ppb O<sub>3</sub>”, and three individuals did not make a clear choice.

290 Bumblebees made landings on artificial flowers in some of the tests conducted  
291 (Figure 7). The results show that more bumblebees landed on artificial flowers  
292 associated with “floral scent from distance 0 at 0 ppb O<sub>3</sub>” than on artificial flowers  
293 associated with “clean air” (paired t-test,  $P=0.04$ ) (Figure 7A). More bumblebees  
294 landed on artificial flowers associated with “floral scent from distance 3 at 120 ppb O<sub>3</sub>”  
295 than on artificial flowers associated with “clean air”, but the difference was not  
296 significant (paired t-test,  $P=0.08$ ) (Figure 7B). Finally, more bumblebees landed on  
297 artificial flowers associated with “floral scent from distance 0 at 120 ppb O<sub>3</sub>” than on  
298 artificial flowers associated with “floral scent from distance 3 at 120 ppb O<sub>3</sub>” (paired t-  
299 test,  $P=0.01$ ) (Figure 7C).

300

## 301 **DISCUSSION**

### 302 **Quantitative and qualitative changes in floral scents after exposure to ozone**

303 The concentrations of floral VOCs were significantly reduced with increasing distance  
304 from source when exposed to ozone enriched ambient air. We started to observe  
305 degradation of the floral volatiles emitted by *B. nigra* at the lower ozone level tested (80  
306 ppb) over a distance of 1.5 m. The highest degradation levels of 25 to 30% were

307 observed at 120 ppb O<sub>3</sub> over a distance of 4.5 m. Ozone degradation of vegetative  
308 VOCs has been previously reported (Pinto *et al.*, 2007a, 2007b, 2010; Blande *et al.*,  
309 2010; Li & Blande, 2015) but, to our knowledge this is the first work to provide  
310 experimental evidence and quantification of floral scent degradation with ozone  
311 exposure. McFrederick *et al.* (2008) previously published a theoretical work modeling  
312 the degradation of three common floral monoterpenes under different concentrations of  
313 ozone and hydroxyl and nitrate radicals, whose predictions are mostly in accordance  
314 with our results. Girling *et al.* (2013) empirically demonstrated that diesel exhaust  
315 fumes, which include oxidant pollutants other than ozone, such as NO<sub>2</sub>, NO, CO and  
316 SO<sub>2</sub>, degrade floral scent volatiles that play relevant roles in the stimulation of  
317 proboscis extension reflex in honeybees. Also, several previous works have examined  
318 the ozone degradation of vegetative VOCs and showed how this can interfere with, or  
319 even disrupt some other ecological interactions of plants (Pinto *et al.*, 2007a, 2007b;  
320 Blande *et al.*, 2010; Li & Blande, 2015).

321 Individual VOCs in the blend of floral volatiles showed varying degrees of  
322 degradation, which are explained by their different reactivities with ozone (Atkinson *et*  
323 *al.*, 1995; Atkinson & Arey, 2003). The range of different reaction rates with ozone  
324 displayed by VOCs in the floral scent blend suggests that ozone pollution will induce  
325 changes in the relative composition of floral blends and that these changes will increase  
326 with increasing distance from the volatile source. In fact, we detected some changes in  
327 the relative composition of terpenes in the floral scent with increasing ozone  
328 concentration and distance, although they were not found to be significant probably due  
329 to low statistical power (Figure 5).

330

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

332 Our results on the behavioral responses of *B. terrestris* clearly indicate a reduction in  
333 orientation toward floral scent cues after they have been exposed to ozone. *B. terrestris*  
334 displayed a clear orientation bias towards unaltered floral scent over clean air (Figure  
335 6A) and there were significantly more landings on the artificial flowers associated with  
336 that scent (Figure 7A). This observation confirmed the usage of floral scent cues by *B.*  
337 *terrestris* and also set a baseline observation for our behavioral arena. We later  
338 compared the responses of *B. terrestris* to floral scent exposed to 120 ppb ozone over  
339 the longest distance of 4.5 m against clean air and pollinators showed no preference for  
340 either of the two options (Figures 6B, 7B). This clearly suggests that exposure of floral  
341 scent to high ozone concentrations led to a loss in attractiveness of the floral scent to  
342 pollinators. Finally, we compared the responses of *B. terrestris* presented with a choice  
343 of floral scent mixed with 120 ppb ozone at distances of 0 m and 4.5 m through the  
344 reaction chamber, and observed that pollinators clearly preferred the scent at the 0 m  
345 distance (Figure 6C) and visited the artificial flowers associated with it more frequently  
346 (Figure 7C), which strongly supports that attraction to floral scent is gradually reduced  
347 with distance under high ozone concentrations.

348 We observed a significant degradation of floral scent cues after exposure to  
349 ozone, which may explain the loss of attractiveness to pollinators. High ozone  
350 concentrations like those tested here may cause a significant reduction in the distance  
351 that floral chemical cues can travel before reaching concentration levels that are below  
352 the olfactory detection limits of pollinators. This may be translated into a significant  
353 reduction in the distance over which floral chemical cues can be utilized by pollinators.  
354 Previous work by Girling *et al.* (2013) demonstrated that primary pollutants in diesel  
355 exhaust can differentially degrade the volatiles emitted by oilseed rape flowers. They



356 additionally showed that removal of the two most reactive compounds from the blend  
357 resulted in a loss of the proboscis extension reflex of conditioned honeybees. Although  
358 the blend modification tested was a little bit more extreme than those encountered upon  
359 natural degradation processes, the removal of those two reactive compounds provides a  
360 strong indication that floral blend alteration has an important impact on foraging  
361 behaviors. In this work, we showed that far more moderate alterations of the entire  
362 blend, not involving the full elimination of any specific component, result in a loss of  
363 attractiveness of the blend to pollinators.

364           Qualitative changes in floral scent composition may lead to disturbance of  
365 pollinator attraction to floral odor plumes (Beyaert & Hilker, 2014). The correct  
366 recognition of plant volatile cues by foraging insects depends not only on the presence  
367 of certain compounds or the magnitude of the whole signal, but also on the ratios of the  
368 compounds that constitute the volatile blend (Bruce *et al.*, 2005). The effects of  
369 qualitative changes in floral scents on the attraction of pollinators may depend on the  
370 reliance of pollinators on innate olfactory preferences and their olfactory learning  
371 capabilities (Cunningham *et al.*, 2004; Schiestl & Johnson, 2013). While specialist  
372 pollinators show innate preferences towards specific blends of volatiles that are typical  
373 of their host plants, generalist pollinators are capable of learning the floral scents of the  
374 plants in the community and associate them with their floral rewards (Raguso, 2008;  
375 Riffell, 2011; Riffell *et al.*, 2013). For this reason, it is important for reward-offering  
376 plants to maintain a good level of reliability in their floral signals for pollinators,  
377 through the maintenance of low levels of variability (Wright & Schiestl, 2009; Knauer  
378 & Schiestl, 2014). Such low levels of variability in floral traits have been postulated to  
379 be beneficial for reward-offering plants (Salzmann *et al.*, 2007). Pollinators promote the  
380 selection of uniformity in the olfactory and visual traits of rewarding flowers, due to the

381 advantages that flower consistency bring to both pollinators (higher foraging efficiency)  
382 and plants (less deposition of heterospecific pollen on the stigmas) (Gegear & Lavery,  
383 2005). The qualitative changes in the relative composition of floral volatile cues caused  
384 by ozone exposure can have significant negative impacts on the correct learning and  
385 recognition of floral olfactive signals by foraging pollinators.

386

### 387 **Implications of floral scent degradation by increasing tropospheric ozone** 388 **concentrations**

389 The increase in tropospheric ozone since the start of the industrial era is estimated to be  
390 around 35% with subtle differences among regions (IPCC, 2001, 2007, 2013). Mean  
391 annual tropospheric ozone concentrations over the mid latitudes of the Northern  
392 Hemisphere currently range between 20 and 45 ppb (Vingarzan, 2004). However, ozone  
393 concentrations are significantly higher in some areas (Kleinman *et al.*, 2002), which can  
394 reach or surpass 120 ppb, the highest ozone concentration that we tested in our  
395 experiments. The effects revealed by our work may be especially relevant for those  
396 regions with high tropospheric ozone concentrations. Many insect species could be  
397 negatively affected by disruption of volatile chemical communication due to ozone  
398 pollution. In the case of pollinator species these effects would have major economic and  
399 ecological impacts. Among the plant communities experiencing the most relevant  
400 effects we may find agricultural lands close to urban areas to be reduced in pollination  
401 efficiency. The most important concerns arising from these results may include reduced  
402 crop productivity and the disruption of several ecological processes related with  
403 pollination in plant communities affected by ozone pollution.

404

## 405 **Conclusions and future perspectives**

406 Our results strongly suggest that ozone can have significant negative effects on  
407 pollinator attraction to flowers. High ozone concentrations in ambient air caused fast  
408 degradation of *B. nigra* floral scent with increasing distance from the scent source,  
409 reducing the range over which flowers can be identified by pollinators. Behavioral tests  
410 conducted with *B. terrestris*, a common and widespread generalist pollinator, confirmed  
411 that ozone concentrations of 120 ppb, which can frequently occur near big urban areas,  
412 can strongly inhibit pollinator attraction to flowers.

413         The effects of ozone on VOC mixtures emitted by plants have been explored in  
414 several studies and the implications for plant communication with other plants,  
415 herbivores and predators have been addressed, but the effect on air concentrations of  
416 floral VOCs has not. Therefore, further experiments to test the effects in other plant  
417 species are warranted. In addition to pollinator response tests, new experiments may  
418 also include estimates of pollination success and fruit/seed production to explore the  
419 effect of ozone exposure and the related changes in floral scent on plant reproduction.

420

421

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423

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429

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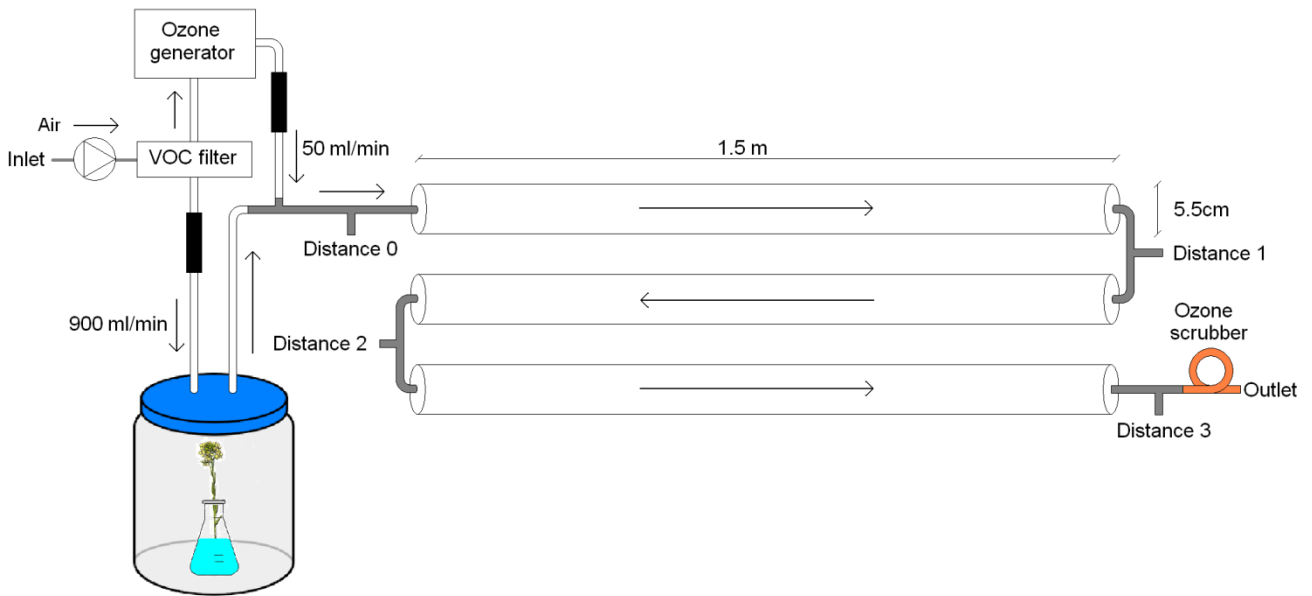
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570 **FIGURE CAPTIONS**

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



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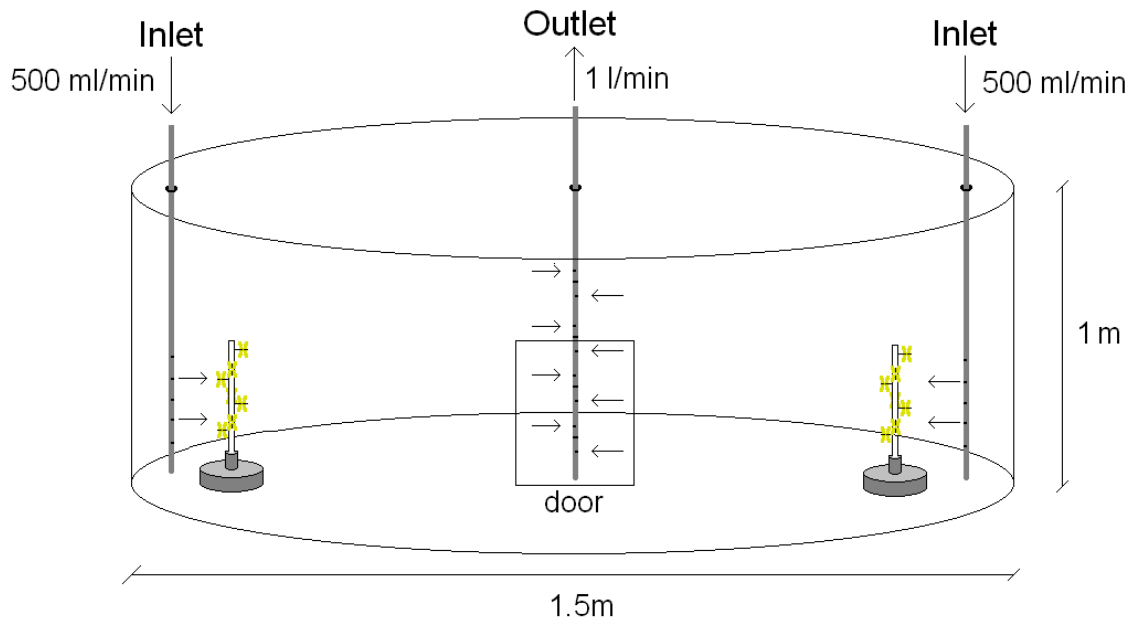
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582 **Figure 2.** Behavioral test chamber. Arrows indicate the direction of air flow.



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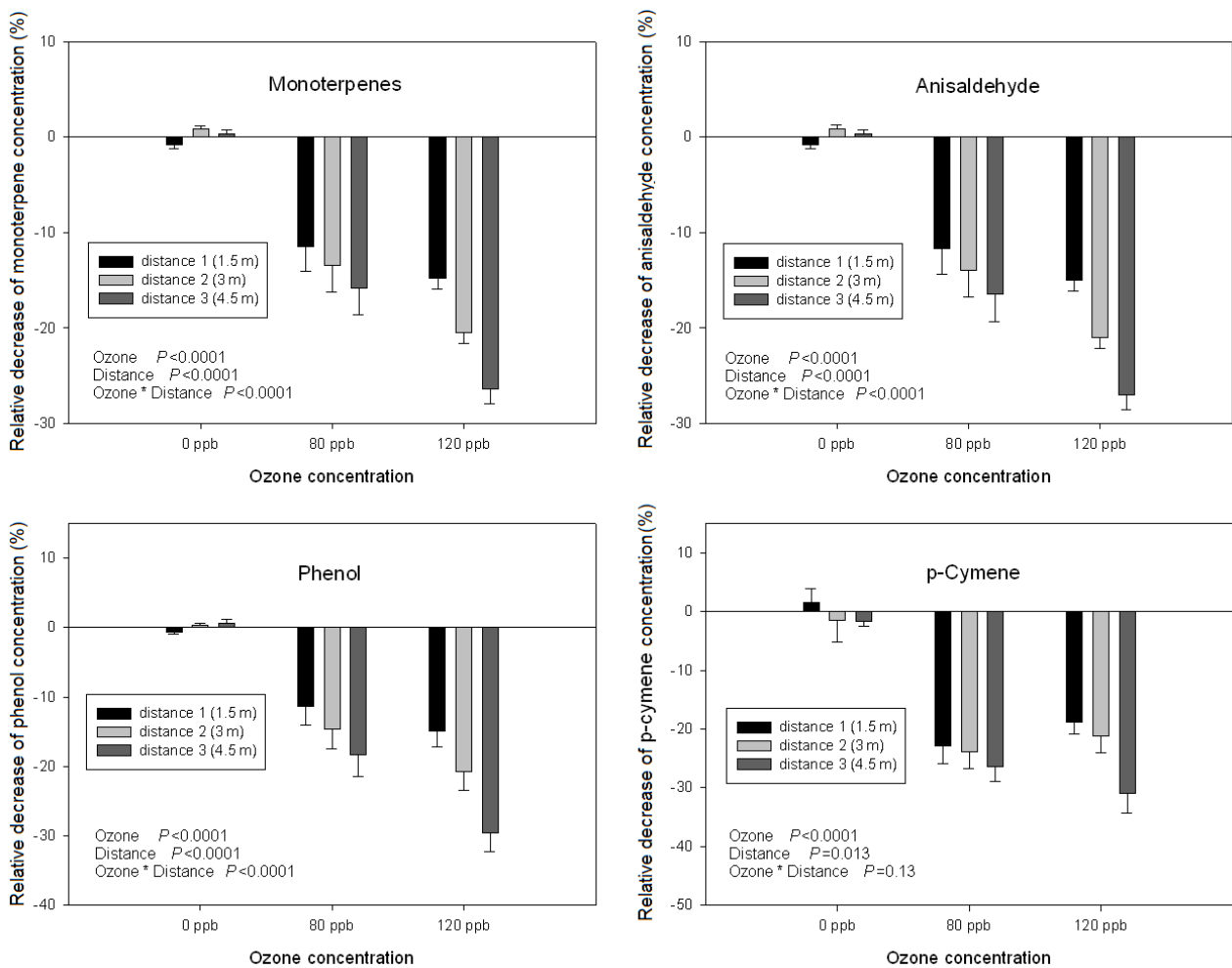
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594 **Figure 3.** Floral scent degradation by ozone. The figure shows the relative decrease in  
 595 monoterpene (m/z 137.133), anisaldehyde m/z (137.1562), phenol (m/z 95.1194) and *p*-  
 596 cymene (m/z 135.1174) concentrations of *Brassica nigra* floral scent exposed to  
 597 different ozone concentrations (0 ppb, 80 ppb, 120 ppb) at different distances from the  
 598 emitter flower source (1.5 m, 3 m, 4.5 m) measured with PTR-TOFF-MS. Error bars  
 599 indicate SEM (n = 8).



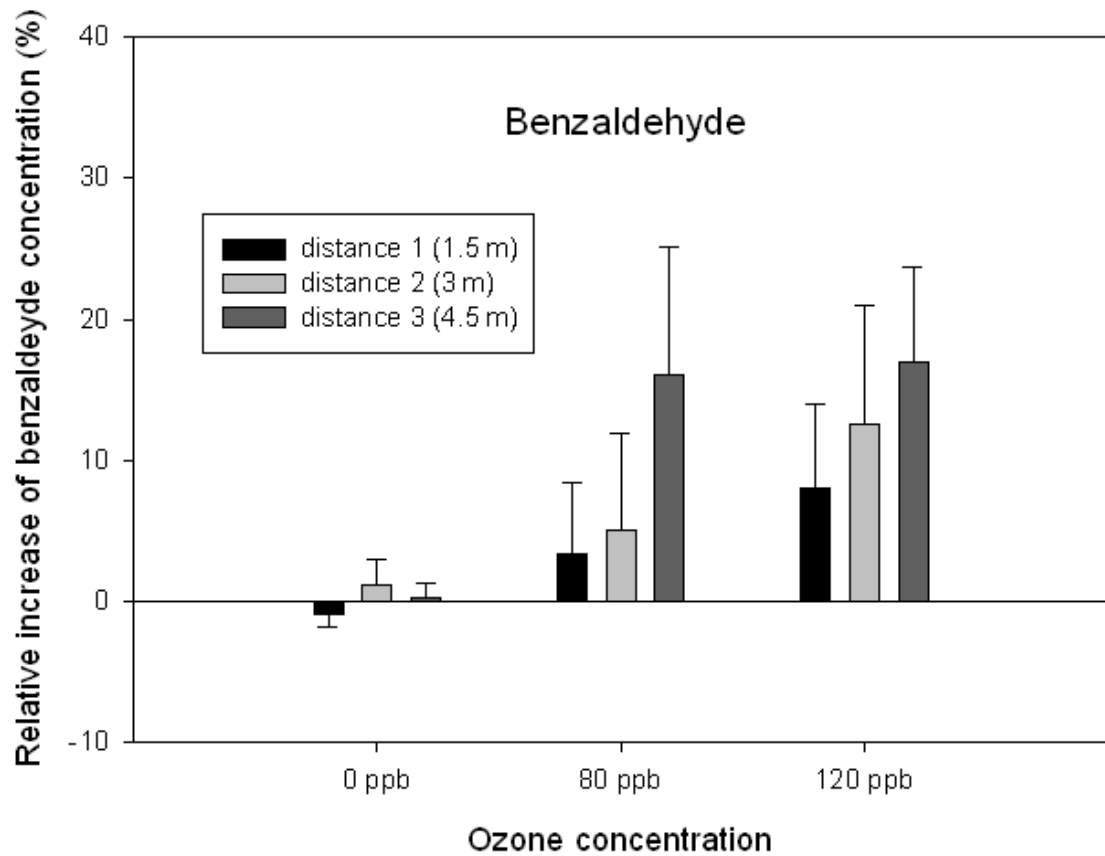
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604 **Figure 4.** Relative increase in benzaldehyde (m/z 107.0497) concentrations of *Brassica*  
605 *nigra* floral scent exposed to different ozone concentrations (0 ppb, 80 ppb, 120 ppb) at  
606 different distances from the emitter flower source (1.5 m, 3 m, 4.5 m) measured with  
607 PTR-TOFF-MS. Error bars indicate SEM (n = 8).



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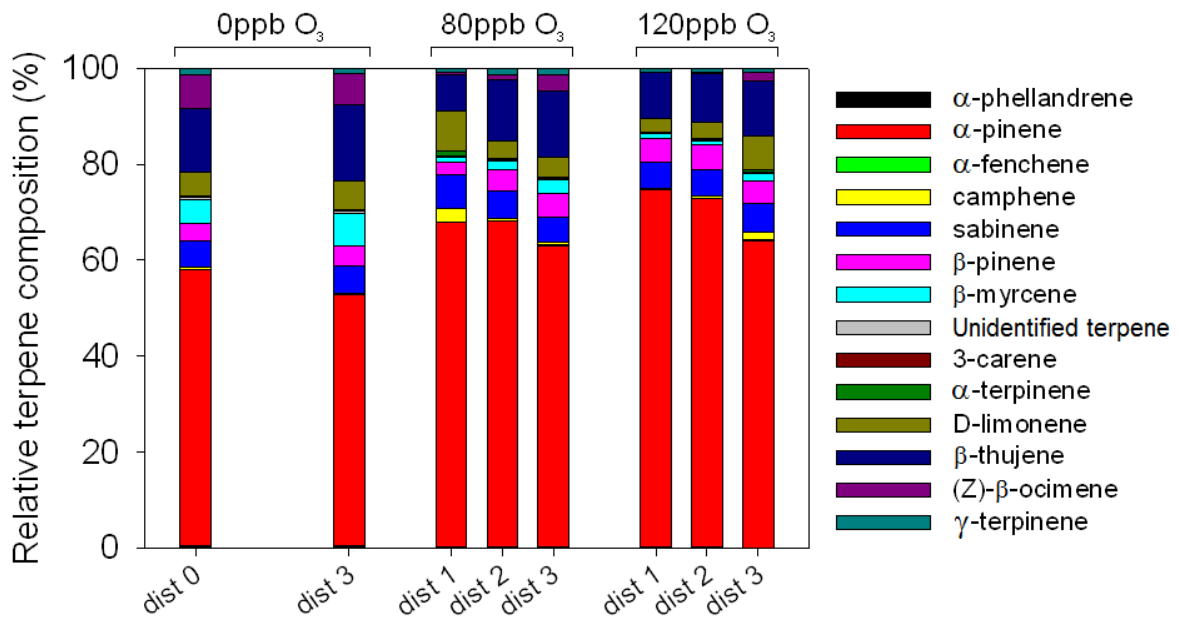
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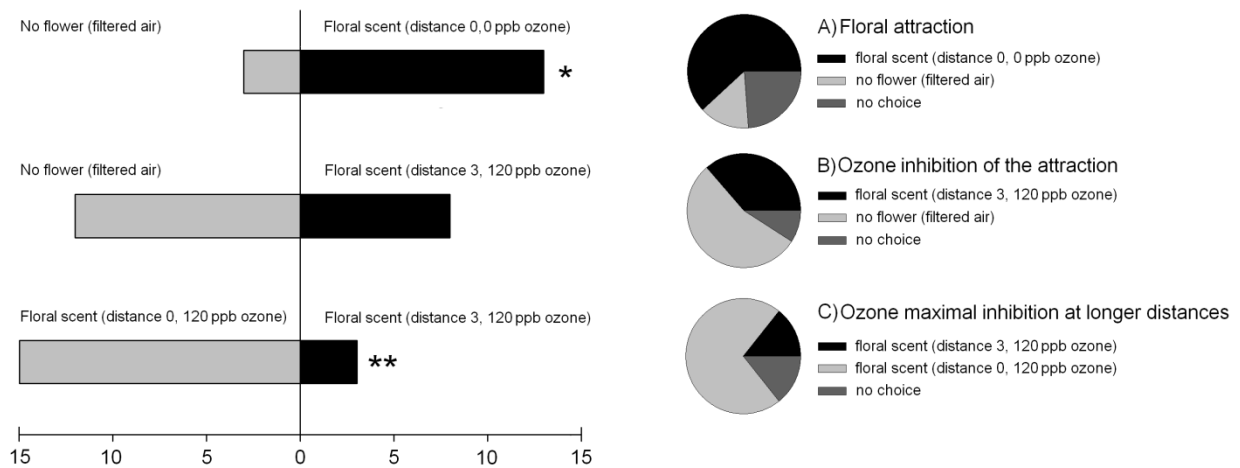
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614 **Figure 5.** Relative floral terpene composition (%) at different distances from scent  
 615 source under different ozone concentrations measured with GC-MS (n = 2, 3, 2, 2, 4, 2,  
 616 2, 4). Changes in the percentage of relative contribution of the different terpene  
 617 compounds to the total terpene emissions were analyzed using general linear models,  
 618 but no significant patterns of change were detected.



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627 **Figure 6.** Pollinator orientation in choice tests comparing: A) floral scent (distance 0 at  
 628 0 ppb O<sub>3</sub>) vs. clean air (filtered air with no flower scent) (*n*=21); B) floral scent  
 629 (distance 3 at 120 ppb O<sub>3</sub>) vs. clean air (filtered air with no flower scent) (*n*=24); C)  
 630 floral scent (distance 0 at 120 ppb O<sub>3</sub>) vs. floral scent (distance 3 at 120 ppb O<sub>3</sub>) (*n*=21).  
 631 Asterisks indicate the level of significance of  $\chi^2$  tests (\**P*<0.05; \*\**P*<0.005).



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642 **Figure 7.** Pollinator visitation to artificial flowers for the behavioral tests comparing: A)  
 643 floral scent (distance 0 at 0 ppb O<sub>3</sub>) vs. clean air (filtered air with no flower scent)  
 644 (*n*=21); B) floral scent (distance 3 at 120 ppb O<sub>3</sub>) vs. clean air (filtered air with no  
 645 flower scent) (*n*=24); C) floral scent (distance 0 at 120 ppb O<sub>3</sub>) vs. degraded floral scent  
 646 (distance 3 at 120 ppb O<sub>3</sub>) (*n*=21). Asterisks indicate the level of significance of paired  
 647 t-tests (\**P*<0.05). Error bars indicate SEM.

648

