

# *The effects of diesel exhaust pollution on floral volatiles and the consequences for honey bee olfaction*

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THE EFFECTS OF DIESEL EXHAUST POLLUTION ON FLORAL VOLATILES AND  
THE CONSEQUENCES FOR HONEY BEE OLFACTION

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1 **Abstract** – There is growing evidence of a substantial decline in pollinators within Europe  
2 and North America, most likely caused by multiple factors such as diseases, poor nutrition,  
3 habitat loss, insecticides and environmental pollution. Diesel exhaust could be a contributing  
4 factor to this decline, since we found that diesel exhaust rapidly degrades floral volatiles,  
5 which honey bees require for flower recognition. In this study we exposed eight of the most  
6 common floral volatiles to diesel exhaust in order to investigate, whether it can affect volatile  
7 mediated plant-pollinator interaction. Exposure to diesel exhaust altered the blend of common  
8 flower volatiles significantly: myrcene was considerably reduced,  $\beta$ -ocimene became  
9 undetectable, and  $\beta$ -caryophyllene was transformed into its *cis*-isomer isocaryophyllene.  
10 Proboscis extension response (PER) assays showed that the alterations of the blend reduced  
11 the ability of honey bees to recognize it. The chemically reactive nitrogen oxides fraction of  
12 diesel exhaust gas was identified as capable of causing degradation of floral volatiles.

13

14 **Key Words** - Floral scent compounds, diesel exhaust, nitrogen oxides, scent degradation,  
15 scent recognition, proboscis extension response.

## INTRODUCTION

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Since the winter and spring of 2006-7 there have been reports of elevated colony losses of managed European honey bees (*Apis mellifera*) in the U.S.A., Europe, the Middle East, and Japan (Abrol 2012; Oldroyd 2007). Furthermore, there is growing evidence of substantial losses of many wild pollinator species worldwide, mainly within Europe and North America (Biesmeijer et al. 2006; Potts et al. 2010). Declines of managed honey bees and wild pollinators may have serious implications, particularly because global food security is considered to be dependent on animal pollination (Abrol 2012). Even though most staple food crops do not require insect pollination (Ghazoul 2005), 35% of the world crop production for human food depends on pollinators (Klein et al. 2007). Those pollinated crops are particularly important for our food diversity and add nutritional value to our diet (Steffan-Dewenter et al. 2005). Some crops, such as oilseed rape (*Brassica napus*), are considered self-fertile (Free 1993), but insect pollination can strongly contribute to increased yield and market value (Bommarco et al. 2012). About 73% of cultivated crop varieties are pollinated by some type of bee, with the European honey bee dominating crop pollination worldwide (Abrol 2012). The drivers behind the global pollinator decline are likely to be multifactorial and include fragmentation and loss of habitat, increased pesticide use, decreased resource diversity, alien species, spread of pathogens, and climate change (Epstein et al. 2013; Kerr et al. 2015; Oldroyd 2007; Potts et al. 2010, Vanbergen et al. 2013). However, it is unlikely that this is a definitive list, and it is, therefore, important to investigate additional potential stressors that could result in negative effects on bee fitness.

Bees use mixtures of olfactory and visual stimuli to find suitable host plants (Dötterl and Vereecken 2010). They rely mainly on olfactory cues during their initial foraging bouts, and visual cues become more important in host-plant location as bees gain more experience (Dobson 1994). However, floral scent remains an important stimulus for experienced bees,

42 since it allows them to discriminate between rewarding and non-rewarding flowers (Dobson  
43 2006; Wright and Schiestel 2009).

44 Ozone or reactive species, such as hydroxyl and nitroxyl or nitrate radicals, readily  
45 react with volatile organic compounds (VOC) (Atkinson and Arey 2003; Calogirou et al.  
46 1999), which contribute to floral scent and could, therefore, impact upon VOC-mediated  
47 plant-insect interactions. McFrederick et al. (2008) modelled the dispersion of three common  
48 floral scent compounds (linalool, myrcene, and  $\beta$ -ocimene) under different air pollution  
49 scenarios and concluded that increasing pollution levels may impair the recognition of floral  
50 scents by pollinators.

51 In an earlier study we were able to show that diesel exhaust alters the VOC  
52 composition of a synthetic floral odour blend (Girling et al. 2013) designed to mimic the  
53 oilseed rape (OSR) cultivar *Brassica napus*, cv. Topas. It consisted of eight compounds,  
54 which are behaviourally active in honey bees, mixed in their naturally occurring ratios (Blight  
55 et al. 1997). The exposure of the OSR blend to diesel exhaust rendered two compounds  
56 undetectable, which significantly reduced the ability of honey bees to recognize the altered  
57 floral scent (Girling et al. 2013). Whether such changes deleteriously affect honey bee  
58 foraging or foraging by other pollinators that utilise these compounds is currently unknown.  
59 Three of the volatile compounds in OSR are floral scent compounds that occur in more than  
60 50% of seed plants (reviewed by Knudsen et al. 2006).

61 In order to investigate further the effects of diesel exhaust emissions on floral VOCs  
62 and to study whether such effects could be a more widespread phenomenon, we exposed eight  
63 of the 12 most common floral scent compounds (Knudsen et al. 2006) to diesel exhaust, none  
64 of which are found in OSR, mixed in equal amounts. To investigate if bees that were  
65 conditioned to our floral volatile blend were able to still recognize it after they underwent the  
66 alterations caused by diesel exhaust, we used the classical conditioning of the proboscis  
67 extension reflex (PER; Bitterman et al. 1983; Kuwabara 1957). Additionally, we exposed all

68 eight common floral scent compounds individually to diesel exhaust with the intention of  
69 determining their possible fate in a diesel exhaust polluted environment. In order to confirm  
70 the general mechanism behind the alteration of floral scent components in such an  
71 environment, we also exposed all compounds to various concentrations of NO<sub>x</sub>, since that  
72 was the observed mechanism behind the floral scent degradation in our earlier study (Girling  
73 et al 2013).

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76

## METHODS AND MATERIALS

77

78 *Floral Volatile Blend.* Based on a review by Knudsen et al. (2006) on the diversity and  
79 distribution of floral scent, we selected eight common floral compounds that occur in more  
80 than half of all the families of seed plants:  $\beta$ -Pinene, myrcene, limonene,  $\beta$ -ocimene,  
81 benzaldehyde,  $\beta$ -caryophyllene, methyl salicylate, and benzyl alcohol (Table 1). We mixed all  
82 eight compounds in equal amounts to produce a common floral volatile (CFV) blend.

83

84 *Floral Volatile Exposures.* For the exposures the general procedure was to place 1 $\mu$ L of CFV  
85 blend on a filter paper (2.1cm circle, grade 3MM; Whatman plc, Maidstone, UK) and a 4.5cm  
86 stir bar in a 1000mL glass bottle (VWR International Ltd., West Sussex, UK). To assess the  
87 influence of UV light the tests were replicated in both clear and amber glass bottles. The CFV  
88 blend was exposed to either ambient air or diesel exhaust at room temperature. The ambient  
89 air treatment consisted of a bottle of air collected from the laboratory. Diesel exhaust was  
90 collected into the bottle from the exhaust pipe of a Suntom SDE 6500 E diesel generator  
91 (Fuzhou Suntom Power Machinery Co., Ltd. Fuzhou, China) for 3min at a flow rate of  
92 1L/min. Bottles were sealed with two layers of Parafilm M<sup>®</sup> (Bemis Flexible Packaging,  
93 Oshkosh, WI, USA) and a lid. The lid contained a 1mm bore hole in its centre to allow



94 insertion of the solid-phase microextraction (SPME) fibre holder for the collection of  
95 volatiles. The bottle content was mixed with a stir bar at 300rpm. The content of the bottle  
96 was sampled after 1min, 30min, 1h and 2h. At each sample time point the SPME fibre was  
97 extended into the bottle and exposed to the volatiles for 5min. The SPME fibre was then  
98 inserted into the injector of the gas chromatograph linked to the mass spectrometer (GC/MS)  
99 and allowed to desorb for 10min. This 10min desorption period was sufficient to clean the  
100 SPME fibre so that the same fibre could be used to collect VOCs from the same bottle and  
101 filter paper at a later time point. After the collection of VOC at all four time points the bottle  
102 was cleaned with hexane, dried under nitrogen gas, and left in the fume hood overnight to  
103 remove the hexane residue. Each of the air treatment and bottle type combinations were  
104 repeated five times at room temperature for each of the two floral blends.

105

106 *Floral Blend GC/MS Analyses.* After each VOC adsorption period the SPME fibre assembly  
107 (65µm PDMS/DVB, fused silica, 24Ga, manual holder; Supelco, Bellefonte, PA, USA) was  
108 injected into a Hewlett Packard HP6890/5972A GC/MS at an injection port temperature of  
109 250°C and a split ratio of 10:1. The GC/MS was equipped with a SPME injection sleeve (I.D.  
110 0.75mm, Supelco) and HP-Innowax column (I.D. 0.25mm, length 30m; film thickness  
111 0.25µm; Agilent Technologies, Santa Clara, CA, USA). The helium carrier gas flow was set  
112 at 1.0mL/min, and the oven temperature was held for 2min at 50°C, increased to 70°C by 5°C  
113 per min and then ramped up to 240°C by 10°C/min.

114 Peaks were identified using the same standards used to produce the floral volatile  
115 blends (Table 1). Isocaryophyllene the rearrangement product of β-caryophyllene, was  
116 identified based on catalytic conversion of β-caryophyllene in clove oil following US patent  
117 3621070 (Rachlin 1971).

118

119 *Preparation of Bees.* Honey bees were kept on the University of Southampton campus in an  
120 apiary (50° 56' 10"N, 1° 23' 39"W). During the summers of 2012 and 2013, on days when  
121 bees showed high activity, 30-35 returning forager bees were caught at their hive entrance  
122 between 14:00-16:00 GMT. Bees were caught in Sterilin 30mL universal containers (Sterilin  
123 Limited, Cambridge, UK) and kept individually. They were immobilized on ice and  
124 transferred into blue 1mL pipette tips that were cut to resemble the PER tubes used by  
125 Bitterman et al. (1983) and harnessed to it with two strips of cloth tape (tesa<sup>®</sup> extra Power  
126 Perfect). Harnessed bees could freely move their antennae, mouth parts and forelegs (Fig. 1).  
127 The PER tubes containing bees were kept in Eppendorf centrifuge tube racks placed inside  
128 plastic boxes with perforated lids. The bottom of the box was lined with wet tissue to provide  
129 humidity and prevent the bees from desiccating. The bees were fed with a 30% sucrose  
130 solution between 16:00-18:00 and kept in an environmentally controlled room at 20°C  
131 overnight. PER assays were conducted the next morning for which bees were equally divided  
132 into groups of 7-10 bees (depending on overnight survival).

133

134 *Proboscis Extension Response Assay.* One harnessed bee at a time was placed in the  
135 experimental arena (W×D×H= 60cm×45cm×55cm) 3cm in front of an odour delivery system.  
136 Behind the bee, an extraction fan removed the odour from the arena in order to avoid a build-  
137 up of any stimuli. The odour delivery system was custom-built. It allowed for a constant  
138 stream of fresh air in the arena through Teflon tubing which flowed into a glass tube. The  
139 conditioning stimuli were delivered through a three channel system that ended in the same  
140 glass tube as the fresh air, before it discharged into the arena. All three channels consisted of  
141 electronic valves and Teflon tubing connected to 10mL glass tubes, in which the stimuli were  
142 placed on a piece of filter paper. One channel served as the control (clean air), which was  
143 always open when no volatiles were delivered. In order to deliver scented air, the control  
144 channel was switched. The airflow passing through the odour delivery part of the system was

145 set at 300mL/min and the fresh air at 200mL/min air. Hence, the bees were constantly  
146 exposed to an airflow of 500mL/min. The following timeline applied to the PER assay: A bee  
147 was allowed to adapt to the experimental arena for 10s, then was presented with the  
148 conditioning stimulus (CS) for 10s. Five seconds after the onset of CS, the antennae of the bee  
149 was touched with a cocktail stick that was dipped in 30% sucrose solution (unconditioning  
150 stimulus (US)). Upon extension of her proboscis, the bee was allowed to feed on the sucrose  
151 solution for the remaining part of the 10s US period. The bee was then removed from the  
152 arena. Thirty seconds was allowed between bees to give the extraction fan additional time to  
153 clear the experimental area from any remaining odours.

154         The bees were conditioned six times to the CFV blend. During these conditioning  
155 trials most bees learned to associate the sugar reward with the odour stimuli. Bees which had  
156 learned to extend their proboscis in response to the odour stimuli by the sixth conditioning  
157 trial were used in the subsequent recognition trials. The recognition trial resembled the  
158 conditioning trial but without the use of the US. Its purpose was to test the proboscis response  
159 of the bees to the original flower blends and to three different test stimuli for each blend  
160 (Table 2). The test stimuli were based on the original blend but some chemical compounds  
161 were omitted based on our results from the floral volatile exposure experiment. Extension of  
162 the proboscis in response to the test stimulus was rated as a positive recognition. The PER  
163 assay was repeated until  $n \geq 25$  was achieved for each test group of bees. The results show the  
164 percentage of bees that had successfully learned to recognize the floral odour blends and then  
165 responded to either of the test stimuli.

166  
167 *Fate of Individual Floral Volatiles.* The floral volatile exposure method described above was  
168 altered slightly when investigating the fate of all individual compounds as these analyses were  
169 conducted on a replacement Agilent Technologies 7890B/5977A GC/MS. Because of its  
170 higher sensitivity only 0.2 $\mu$ L of each compound was placed on a filter paper and transferred

171 into an amber bottle with either ambient air or diesel exhaust. The content of the bottle was  
172 sampled after 30min, and the SPME fibre was exposed to the volatile for 1min. The SPME  
173 fibre was then inserted into the injection port of the GC/MS at 250°C and a split ratio of 10:1.  
174 The GC/MS was equipped with a SPME injection sleeve, a HP-Innowax column (I.D.  
175 0.25mm, length 30m; film thickness 0.25µm) and a retention gap (I.D. 0.18mm, length 4.1m,  
176 film thickness 0µm; both Agilent Technologies, Santa Clara, CA, USA). The helium carrier  
177 gas flow and oven temperature programme remained the same as for the floral blend analyses  
178 described above.

179

180 *Diesel Exhaust Measurements.* In order to determine the concentration of toxic and reactive  
181 gases in our diesel exhaust we used a Wolfsense TG501 probe equipped with O<sub>2</sub>, NO, NO<sub>2</sub>,  
182 SO<sub>2</sub>, and CO photochemical sensors (GrayWolf Sensing Solutions, Shelton, CT, USA).  
183 Diesel exhaust was pumped through the probe at a flow rate of 1L/min by attaching a  
184 calibration hood. Measured values were logged every 30s. Five 3min measurements were  
185 taken to mimic the diesel exhaust collection for floral volatile exposures.

186

187 *Influence of Nitrogen Oxides.* For the exposures to nitric oxides (NO<sub>x</sub>) a filter paper with 1µL  
188 of the CFV blend and a stir bar were added to an amber 1000mL bottle. Nitrogen oxides were  
189 produced by reducing nitric acid with elemental copper, which resulted in a 1:1 ratio of NO  
190 and NO<sub>2</sub>, which was confirmed by the Wolfsense TG501 probe. Concentrations of 20ppm,  
191 2ppm, 0.2ppm per bottle were achieved by using gas tight syringes and volumetric  
192 calculations. The bottle was closed with two layers of Parafilm M<sup>®</sup> and a lid. The content was  
193 allowed to stir for 30min at 300rpm before the SPME fibre was extended into the bottle.  
194 SPME exposure and the GC/MS method were the same as for floral blend volatile exposures.

195

196 *Statistical Analysis.* As we found that UV light had no statistically significant effect on the  
197 composition of volatiles in our experiments, we pooled the data gained from exposures in  
198 amber and clear glass bottles. Due to the large number of samples for the floral volatile  
199 exposures, it was necessary to use more than one SPME fibre, which did not allow  
200 comparative statistical analyses based on peak area according to the SPME guidelines of the  
201 journal. However, the SPME fibres used were from the same lot, and the peak area data of the  
202 floral volatile exposures had less variance than that of the NO<sub>x</sub> exposure data that was  
203 collected using a single SPME fibre. Therefore, we plotted the peak areas as bar graphs with  
204 95% confidence interval error bars, which provides a graphical display of the significance of  
205 differences of the CFV blend when exposed to either ambient air or diesel exhaust (Fig. 6,  
206 online supplementary material).

207         The PER assay data was analysed by comparing the recognition of each test stimuli to  
208 the response of bees to the complete CFV blend. Pairwise comparisons were made using  $\chi^2$ -  
209 tests. The criterion for significance was corrected after Bonferroni:  $\alpha' = \alpha/k$ , where k is the  
210 number of comparisons.

211         The influence of NO<sub>x</sub> on the CFV blend was measured with the same SPME fibre, so  
212 we were able to directly compare the effect of the different NO<sub>x</sub> concentrations on the  
213 abundance of compounds. The peak area data was analysed with a MANOVA followed by  
214 univariate ANOVAs for each compound and Tukey post-hoc tests for comparing the  
215 influence of different NO<sub>x</sub> concentrations on the single compounds. All statistical analyses  
216 were conducted with SPSS 20.0 for Windows (IBM Corporation, Armonk, New York, USA).

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## RESULTS

220

221 *Floral Volatile Exposures.* At the one minute time point of diesel exhaust exposure of the  
222 CFV blend the compounds myrcene, *Z*- and *E*- $\beta$ -ocimene were drastically reduced, and  
223  $\beta$ -caryophyllene started to be transformed into its *cis*-isomer, isocaryophyllene (Fig. 2). After  
224 30min, when all compounds in the mixture had reached equilibrium in the glass bottle,  
225 myrcene remained strongly reduced, whereas *Z*- and *E*- $\beta$ -ocimene (assignment of geometrical  
226 isomers followed Babushok et al. 2011) became undetectable, and  $\beta$ -caryophyllene was  
227 completely transformed into isocaryophyllene.

228

229 *Proboscis Extension Response Assays.* The recognition assay for the CFV blend revealed that  
230 the PER response of the honey bees was significantly influenced by the test stimuli  
231 composition. The original CFV blend was recognized by 93% of all bees in the absence of a  
232 sugar reward (Fig. 3). The CFV blend without  $\beta$ -ocimene (CFV-1) was still recognized by  
233 76% of bees compared to the original blend, which is non-significant ( $\chi^2_{(1)} = 2.928$ ,  $P =$   
234  $0.092$ ). Omission of myrcene (CFV-2) had a significant effect on the bees' PER response, and  
235 only 37% still recognized the test stimuli ( $\chi^2_{(1)} = 18.941$ ,  $P < 0.001$ ). The test stimuli which  
236 lacked  $\beta$ -ocimene and myrcene (CFV-3) was recognized by 39% of the bees, which is  
237 significantly lower than the recognition of the full CFV blend ( $\chi^2_{(1)} = 17.923$ ,  $P < 0.001$ ).

238

239 *Fate of Individual Floral Volatiles.* Investigation on the effect of diesel exhaust on each single  
240 floral volatile showed that  $\beta$ -caryophyllene was transformed into its *cis*-isomer, confirming  
241 the results that we had seen when we exposed the complete CFV blend to diesel exhaust.

242 These further investigations revealed that the  $\beta$ -caryophyllene standard sample also contained  
243  $\alpha$ -copaene and  $\alpha$ -humulene as impurities (verified by standard injection). The amount of both  
244 of them was also drastically reduced after being exposed to diesel exhaust (see supplementary  
245 material, Fig. 6), however, we were unable to identify any degradation products. Similarly, we

246 could not trace the fate of myrcene and  $\beta$ -ocimene, the two acyclic monoterpenes in the CFV  
247 blend. Benzyl alcohol was partly oxidized to benzaldehyde.

248  
249 *Diesel Exhaust Measurement.* The averages of all measurements of all toxic and reactive  
250 gases  $\pm$  S.E. acquired with the Wolfsense TG501 probe are given in Table 3. Carbon  
251 monoxide represented the biggest fraction of the emission gases. Within the  $\text{NO}_x$  fraction, NO  
252 and  $\text{NO}_2$  were produced approximately at a ratio of 1:1. No sulphur dioxide was detected  
253 which is most likely due to the fact that we used low sulphur diesel.

254  
255 *Influence of Nitrogen Oxides.* The abundances of myrcene, *Z*- $\beta$ -ocimene, *E*- $\beta$ -ocimene,  $\beta$ -  
256 caryophyllene, and benzyl alcohol were altered significantly by  $\text{NO}_x$  (Table 4). In particular,  
257 the abundances of myrcene, *Z*- $\beta$ -ocimene, *E*- $\beta$ -ocimene,  $\beta$ -caryophyllene, and benzyl alcohol  
258 were significantly reduced when exposed to 20ppm  $\text{NO}_x$  (Fig. 4). The conversion of  $\beta$ -  
259 caryophyllene into isocaryophyllene began at a concentration of 0.2ppm  $\text{NO}_x$ .

260

261

262

## DISCUSSION

263

264 Exposure to diesel exhaust led to a reduction of myrcene in the CFV blend,  $\beta$ -ocimene  
265 disappeared, and  $\beta$ -caryophyllene was converted into its *cis*-isomer, isocaryophyllene.

266 Investigations on nitration mechanisms have shown that the reaction of unsaturated  
267 compounds with nitrogen dioxide, which is abundant in diesel exhaust, can lead to *cis/trans*-  
268 isomerization (Augusto et al. 2002; Titov 1963). In the blend used for the behavioural  
269 recognition assays we omitted myrcene and  $\beta$ -ocimene. The lack of  $\beta$ -ocimene did not  
270 influence the recognition of the test stimulus significantly. When myrcene was absent,  
271 recognition decreased significantly to only 37%. Similarly, when both compounds were

272 missing 39% of honey bees recognized the test stimuli, suggesting that the absence of  
273 myrcene predominantly contributes to the lack of recognition. Similarly, Reinhard et al.  
274 (2010) identified myrcene as a key odorant and ocimene as a non-key odorant in the  
275 recognition of complex scent mixtures by honey bees. Isocaryophyllene was not available for  
276 the behavioural assays, however, we hypothesise that the change from  $\beta$ -caryophyllene to  
277 isocaryophyllene could also have changed the insects' odour perception of the CFV blend.  
278 Geometric isomers may differ in their quality of odour (Roderick 1966), e.g. to humans, the  
279 cyclic sesquiterpene (*E*)- $\gamma$ -bisabolene smells soapy and spicy, whereas the odour of (*Z*)- $\gamma$ -  
280 bisabolene is described as fatty and woody (Kjeldsen et al. 2003). The discrimination of  
281 geometric isomers by insects has been mainly studied in the context of straight chain sex-  
282 pheromones of moths (e.g. Klun et al. 1973; Naka et al. 2013; Silverstein and Young 1976).

283         Investigations into the fates of individual flower volatiles could not detect substantial  
284 amounts (if any) of degradation products of the acyclic terpenes myrcene and  $\beta$ -ocimene. On  
285 their oxidative way to carbon dioxide, most biogenetic volatiles pass acetone and  
286 formaldehyde (Atkinson and Arey 2003), which, due to their low boiling points, would have  
287 been difficult to find by our analytical method. However, primary oxidation products of  
288 myrcene such as 4-methylene-5-hexenal and of  $\beta$ -ocimene such as 4-methyl-3,5-hexadienal  
289 (Lee et al. 2006, Reissell et al. 2002) should have been reliably registered.

290         Each of the floral scent compounds used in our study that degraded when exposed to  
291 diesel exhaust also occur in numerous other plant families:  $\beta$ -caryophyllene in 52% (46  
292 families),  $\beta$ -ocimene in 71% (*E*- $\beta$ -ocimene in 64 families and *Z*- $\beta$ -ocimene in 44 families),  
293 and myrcene in 70% (63 families) of all investigated seed plant families (Knudsen et al.  
294 2006). Half of the highly ubiquitous flower scent compounds that we tested experienced  
295 significant reduction as a result of exposure to diesel exhaust, indicating that it has the  
296 potential to disrupt and modify plant floral volatile signalling at a broad scale.



297 Diesel exhaust emissions have received considerable attention due to their health risks  
298 and environmental concerns, the main focus has been on particulate matter and NO<sub>x</sub>  
299 (Setiabudi et al. 2004), which are considered to be the main air pollutants of diesel exhaust  
300 together with carbon monoxide and hydrocarbons. The NO<sub>x</sub> concentrations of our diesel  
301 exhaust were approximately 20ppm for both NO and NO<sub>2</sub>. When we exposed the floral  
302 volatiles to 20ppm NO<sub>x</sub> we observed that it had a similar effect as diesel exhaust: β-  
303 caryophyllene was turned into isocaryophyllene, the amount of myrcene was reduced, β-  
304 ocimene was reduced as well, but still detectable, whereas it had completely disappeared  
305 when exposed to diesel exhaust. In contrast to oxygenation involving oxygen species, radical  
306 reactions induced by NO<sub>x</sub> appear to be responsible for the majority of the changes observed in  
307 our CFV blend when exposed to diesel exhaust. In addition, the particulate matter in diesel  
308 exhaust may have yet unknown adsorptive and catalytic properties. Because of their isoprene  
309 (butadiene) substructure, myrcene and β-ocimene are particularly labile, and radical reactions  
310 may easily cause polymerisation, leading to products that would escape our analytical system.

311 Ambient air quality standards in the EU ([ec.europa.eu](http://ec.europa.eu)) and the US ([www.epa.gov](http://www.epa.gov))  
312 suggest a maximum hourly average NO<sub>2</sub> concentration of 0.1ppm, a concentration at which β-  
313 caryophyllene started turning into isocaryophyllene in this study, and α-terpinene and  
314 phenylacetaldehyde were already significantly degraded, in our previous study. Furthermore,  
315 in 2013 the UK Supreme Court declared that the nitrogen dioxide limits are regularly  
316 exceeded in 16 zones across the UK (European Commission, press release).

317 Ambient NO<sub>x</sub> levels can exhibit strong diurnal patterns, with maximum values in the  
318 early morning and another increase in the late afternoon (Fuentes et al. 2007), following  
319 periods of high traffic. Fluctuating NO<sub>x</sub> levels will change the ratio of compounds in floral  
320 scent throughout the course of the day (McFrederick et al. 2008). Additionally, high NO<sub>x</sub>  
321 levels lead to increased ozone levels (Fehsenfeld et al. 1992), which may further contribute to  
322 the degradation of floral scent. Changing floral scent of a flower can cause floral-constant

323 pollinators, like honey bees, to reject the flower (Wright and Schiestl 2009), which may  
324 impact on pollination efficiency. To date, there is only little research on how air pollution  
325 directly impedes on plant-insect interactions. Fuentes et al. (2013) found that ozone pollution  
326 obstructs host finding in the cucumber beetle (*Acalymma vittatum*). Pinto et al. (2007a; 2007b;  
327 2008) investigated the impacts of ozone on tritrophic interactions under both laboratory and  
328 field conditions. They showed that parasitoids are able to use herbivore-induced plant VOCs  
329 to find their hosts in the presence of ozone, but preferred the intact signal over the ozone-  
330 degraded signal. There is also a possibility that the reaction products of floral scent  
331 compounds with ozone and other air pollutants might form new compounds that may serve as  
332 novel signals (McFrederick et al. 2009).

333 Our study demonstrates that NO<sub>x</sub> and/or other components of diesel exhaust are  
334 capable of degrading floral signals and have the potential to alter floral recognition. How this  
335 impacts upon the fitness of pollinators requires further investigations, however it has been  
336 proposed that pollinators in polluted areas may need to spend more time searching for  
337 adequate host plants, which if true could lead to decreased fitness and decreased pollination  
338 rates (McFrederick et al. 2008).

339

340

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470 **Table 1** Information about compound purity, vapour pressure, supplier information and their occurrence in seed  
 471 plant families as reported in Knudsen et al. 2006

<b>Common flower volatile blend</b>				
<b>compound</b>	<b>purity (%)</b>	<b>vapour pressure (mm Hg<sup>-1</sup> at 25°C)</b>	<b>supplier</b>	<b>occurrence</b>
<b>β-Pinene</b>	98	2.9300	Sigma Aldrich <sup>a</sup>	59%
<b>Myrcene</b>	90	2.0900	Sigma Aldrich <sup>a</sup>	70%
<b>Limonene</b>	97	1.9800	Sigma Aldrich <sup>a</sup>	71%
<b>β-Ocimene</b>	≥ 90	1.5590	Sigma Aldrich <sup>a</sup>	71%
<b>Benzaldehyde</b>	99	0.1270	Sigma Aldrich <sup>a</sup>	64%
<b>β-Caryophyllene</b>	≥ 80	0.0130	Sigma Aldrich <sup>a</sup>	52%
<b>Methyl salicylate</b>	≥ 99	0.0343	Fluka <sup>b</sup>	57%
<b>Benzyl alcohol</b>	99	0.0940	Sigma Aldrich <sup>a</sup>	56%

472 <sup>a</sup> Sigma Aldrich (St. Louis, MO, USA), <sup>b</sup> Fluka (Sigma-Aldrich, Buchs, Switzerland)

473 **Table 2** Compound composition of the common floral volatile and the oilseed rape blend and their according test  
 474 stimuli for the proboscis extension response assays

<b>Common flower volatile blend</b>				
<b>compound</b>	<b>CFV</b>	<b>CFV-1</b>	<b>CFV-2</b>	<b>CFV-3</b>
<b>β-Pinene</b>	1 μL	1 μL	1 μL	1 μL
<b>Myrcene</b> <sup>a</sup>	1 μL	1 μL	-	-
<b>Limonene</b>	1 μL	1 μL	1 μL	1 μL
<b>β-Ocimene</b> <sup>b</sup>	1 μL	-	1 μL	-
<b>Benzaldehyde</b>	1 μL	1 μL	1 μL	1 μL
<b>β-Caryophyllene</b> <sup>c</sup>	1 μL	1 μL	1 μL	1 μL
<b>Methyl salicylate</b>	1 μL	1 μL	1 μL	1 μL
<b>Benzyl alcohol</b>	1 μL	1 μL	1 μL	1 μL
<b>dose</b>	8 μL	7 μL	7 μL	6 μL

475 <sup>a</sup> compound reduced by diesel exhaust, <sup>b</sup> compounds erased by diesel exhaust, <sup>c</sup> compound turns into its *cis*-isomer

476 **Table 3** Diesel exhaust gas composition

gas (unit)	mean	S.E.
Oxygen (%)	19.78 ±	0.10
Nitric Oxide (ppm)	19.84 ±	2.27
Nitrogen Dioxide (ppm)	17.54 ±	1.25
Sulphur Dioxide (ppm)	0.00 ±	0.00
Carbon Monoxide (ppm)	226.84 ±	20.47

477



478 **Table 4** ANOVA results for single compounds which abundances were significantly decreased by NO<sub>x</sub> exposure  
479 (also see Fig. 4)

compound	<i>F</i> -value	P-value
Myrcene	$F_{(3,12)} = 11.803$	0.001
<i>Z</i> - $\beta$ -Ocimene	$F_{(3,12)} = 89.226$	< 0.001
<i>E</i> - $\beta$ -Ocimene	$F_{(3,12)} = 96.951$	< 0.001
$\beta$ -Caryophyllene	$F_{(3,12)} = 568.459$	< 0.001
Benzyl alcohol	$F_{(3,12)} = 9.184$	0.002

480

481 **Fig. 1** Harnessed honey bee ready for PER assay

482

483 **Fig. 2** Total ion current (TIC) chromatogram of the common flower volatile under 30min  
484 ambient air and diesel exhaust exposure. Consecutive numbers represent the following  
485 chemical compounds:  $\beta$ -Pinene (1), myrcene (2), limonene (3), *Z*- $\beta$ -ocimene (4a),  
486 *E*- $\beta$ -ocimene (4b), benzaldehyde (5),  $\beta$ -caryophyllene (6a), isocaryophyllene (6b) methyl  
487 salicylate (7), benzyl alcohol (8).

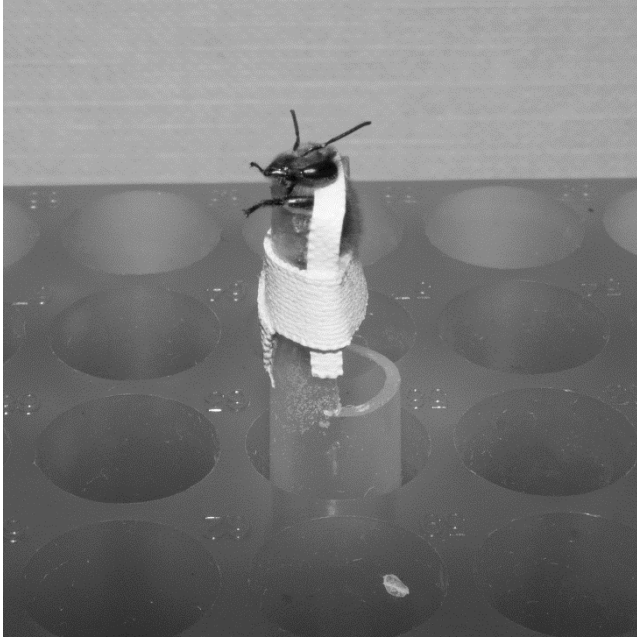
488

489 **Fig. 3** Proboscis extension response assay results representing the ability of honey bees to  
490 recognize the common floral volatile (CFV) blend, CFV blend without  $\beta$ -ocimene (CFV-1),  
491 CFV blend without myrcene (CFV-2), and CFV blend without both (CFV-3). The response to  
492 the test stimuli was compared to the response to the full blend. A Bonferroni correction was  
493 applied, and all significant differences are indicated with an asterisk at a 0.016 level of  
494 significance

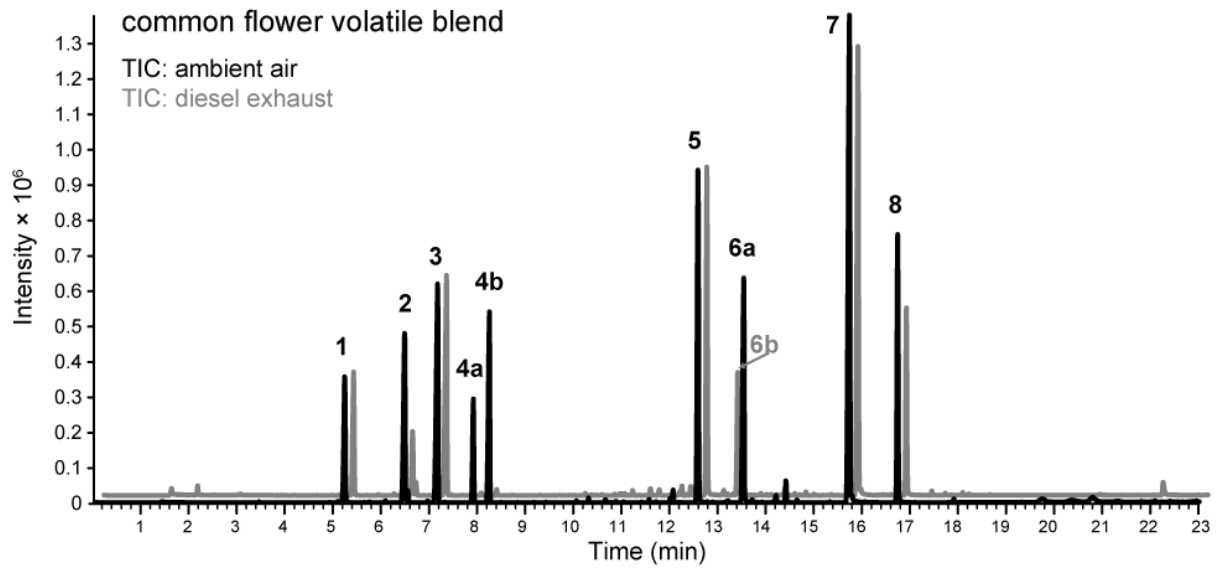
495

496 **Fig. 4** The effect of 30min exposures of different nitrogen oxides ( $\text{NO}_x = \text{NO}$  and  $\text{NO}_2$  at a  
497 1:1 ratio) concentrations on the abundance of all floral compounds used in this study. Bars  
498 represent the average peak area ( $\pm$ S.D). Different lowercase letters indicate a statistically  
499 significant difference.

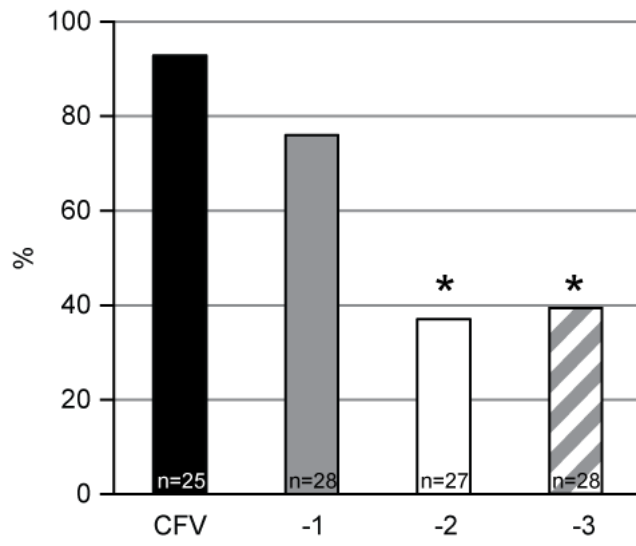
500



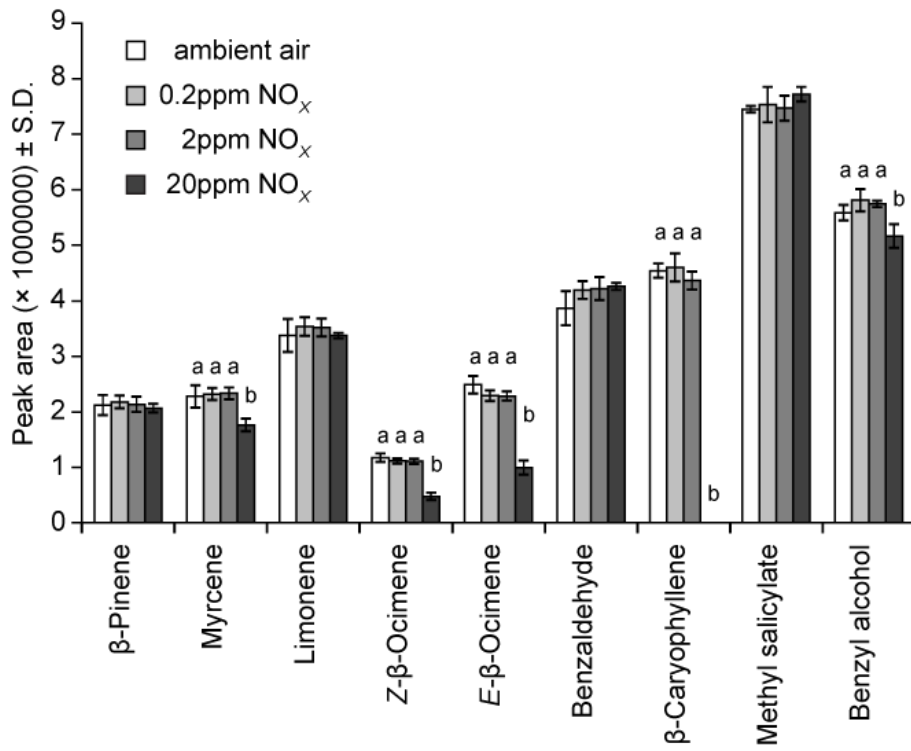
501  
502 Fig. 1



503  
504 Fig. 2  
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509  
510 Fig. 4