Investigating the effects of diesel exhaust and flower color on flower visitation by free-flying honey bees

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3   bees

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36   **Abstract:**

37   Previous laboratory studies have shown that diesel exhaust can differentially degrade the volatile
38   organic compounds (VOCs) that constitute floral odors. Furthermore, in proboscis extension response
39   studies honey bees have been shown to have reduced recognition to these degraded floral odors. In this
40   study, we investigated whether flower odors exposed to diesel exhaust reduce foraging in free-flying
41   bees and if flower color influences bees' behavior. Therefore, we conducted a field study in which
42   honey bees were trained to visit the locations of two arrays of artificial flowers. From the artificial
43   flowers, honey bees were presented with floral VOCs combined with either fresh air or diesel exhaust,
44   through different colored flowers (black, blue, red, and yellow). Honey bee visitation rate did not
45   differ between volatiles delivered with fresh air or with diesel exhaust, suggesting that revisitation of
46   previously rewarding flower patches may be unaffected by air pollution. We also observed a
47   significant interaction between treatment and color: blue flowers were more attractive when volatiles
48   were delivered with diesel exhaust, which was the other way around for red and black and played no
49   role in yellow flowers. Generally, honey bee foraging behavior seemed to be influenced by their
50   previous experiences.

51

2

53 **Statements and Declarations**

54

58

59 **Conflict of interest**

60 The authors declare no conflicts of interest.

61

62 **Data availability**

63 The datasets generated during and/or analyzed during the current study are available from the

64 corresponding author on reasonable request.

65

66 **Contributions**

67 RDG, TAN and GMP conceived the study. IL designed the artificial flower setup and conducted the

68 experiment. IL and LD performed the analysis and wrote the manuscript. CWJ took the UV-VIS

69 measurements and UV pictures. All authors discussed the results and commented on the manuscript.

70

3

Introduction

Managed European honey bees (*Apis mellifera*) provide critical pollination services for the production of a wide variety of flowering agricultural crops worldwide, the yields of which can be limited by insufficient insect pollination (Reilly et al. 2020). Whilst there is evidence that wild pollinators can be more efficient (Breeze et al. 2011; Garibaldi et al. 2013), a large proportion of current agricultural systems rely on honey bees for pollination service provision (Calderone 2012; Garibaldi et al. 2013). Furthermore, evidence suggests that honey bees are also the most important pollinators in natural habitats worldwide (Hung et al. 2017).

Flower location and recognition is a critical step in the provision of pollination services. During this process, honey bees integrate multisensory stimuli such as odor, color, size and shape (Chittka & Raine 2006), but floral color and odor are considered to be the most important cues (Burger et al. 2010). Visual cues such as floral color are mainly used for short-distance orientation (Dafni et al. 1997) but also contribute to flower constancy, during which honey bees visit the same species of flower, even though other flowers are available. For example, Chittka et al. (2001) demonstrated a relationship between bee-subjective color differences and flower constancy. Honey bees have three receptors for color vision: UV, blue and green (Peitsch et al. 1992). Flower-naïve honey bees prefer bee UV-blue (around 410 nm), bee blue and bee green (around 430 nm and 530 nm, respectively) (Giurfa et al. 1995), but bees can be trained to associate rewards with different floral colors (Menzel 1985).

Floral odors act as long-distance attractants and as both landing and feeding cues (Raguso 2008); these odors are comprised of mixtures of volatile organic compounds (VOCs), which can be plant-species specific (Knudsen et al. 2006). They are easily learned and remembered by a range of insect pollinators (Wright & Schiestl 2009), which contributes to foraging success, increased foraging efficiency (Dötterl & Vereecken 2010) and flower constancy in honey bees (Menzel 1999; Giurfa 2007). However, pollinators forage in a range of environments, both urban and rural, and there is growing evidence to suggest that air pollutants common to both rural (e.g. ozone) and urban (e.g. diesel exhaust) areas can react with the VOCs that constitute floral odors. For example, laboratory studies have shown that ozone can interrupt VOCs that are important for plant-plant signaling (Blande

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et al. 2010) and that are used as floral odors (Saunier and Blande 2019). Diesel exhaust can affect the ability of honey bees to detect a floral odor indirectly by differentially degrading individual components of that floral odor (Girling et al. 2013; Lusebrink et al. 2015). Atmospheric chemistry modelling of the reaction kinetics of floral odor VOCs with common air pollutants suggests that VOC degradation could dramatically reduce the distances over which floral odors are detectable (McFrederick et al. 2008). Furthermore, recent field studies have suggested that air pollution can reduce flower visitation by pollinating insects, including honey bees, and therefore the pollination services they provide (Ryalls et al. 2022). In addition, diesel exhaust exposure can have negative effects on the learning behavior of honey bees and reduce their tolerance to additional stress factors (Reitmayer et al. 2019).

Due to changing traffic volumes throughout the day (e.g. rush hours vs. times with less traffic) air pollution from diesel exhaust will vary accordingly. Therefore, if diesel exhaust interferes with the attractiveness of a flower, this effect may vary over time, which in return might influence foraging success, foraging efficiency and flower constancy. Therefore, we investigated the effects of exposing floral VOCs to diesel exhaust on honey bee foraging, using a free-flight field-based assay. Using an artificial flower setup our objective was to evaluate whether floral odor delivered alongside diesel exhaust, emitted by a diesel generator, reduces foraging in free flying honey bees and whether this foraging behavior is influenced by flower color.

Material and Methods

Study site

The experiments took place in a walled garden in Chilworth, Hampshire, United Kingdom (50° 57′ 49.7″N, 1° 25′ 23.2″W), which housed three British Standard National beehives and eight nucleus hives. Bees were trained by luring them to a small petri dish (diameter 6 cm) with 30% sucrose solution at the hive entrance, which was slowly moved to an intermediate spot approximately 20 m

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128 away from the hives and 30 m from the nucleus hives between two specific locations (~10 m apart)

129 within the garden (also see supplementary file 1 for more detail).

130

131 Experimental setup

132 An artificial flower set up capable of emitting synthetic flower volatiles was designed (Fig. 1; for more

133 details see supplementary file 1). Two setups were built each containing four flowers. Each flower was

134 made of a 7 cm long custom-made glassware adapter with a 14/23 insert joint (flower opening) and a

135 straight 6 mm tubing connection (flower stipe). A Perspex® disk was cut, using a laser cutter, to fit into

136 each glass adapter insert. The disk had a center hole to hold a 0.2 mL PCR tube for offering a sugar

137 reward, and eight holes evenly spaced around the center to allow floral volatile emission. Flower

138 petals were made of 2 mm foam sheets in the colors black, blue, red and yellow that were attached

139 around the Perspex® disk. These colors were chosen with the aim of eliciting a range of behavioral

140 preferences; honey bees are attracted to and can distinguish between yellow and blue (Hill et al 1997),

141 red flowers are less preferred but can be perceived by honey bees (Reisenman and Giurfa 2008) and

142 black flowers are rare in nature. The flowers were inserted into a custom-made Perspex® box, which

143 was covered with camouflage netting and housed the volatile delivery system, which consisted of a

144 battery driven pump, a gas washing bottle, an airflow control valve, and tubing. The volatiles were

145 delivered with a flow of 0.5 L/min through each flower.

146 For the experiment we used a VOC blend, which contained eight common floral compounds that occur

147 in more than half of all the families of seed plants (Knudsen et al. 2006). All compounds (purity) were

148 purchased through Sigma Aldrich (St. Louis, Missouri, USA) and added to the blend in equal

149 amounts: β-pinene (98%), myrcene (90%), limonene (98%), β-ocimene (≥ 90%), benzaldehyde (99%),

150 β-caryophyllene (≥ 80%), methyl salicylate (≥ 99%), and benzyl alcohol (99%).

151 Diesel exhaust was pumped from the exhaust pipe of a diesel generator (Suntom SDE 6500 E, Fuzhou

152 Suntom Power Machinery Co., Ltd. Fuzhou, China) through Teflon tubing into 2 bags (~20 L each),

153 which were made by sealing off a polyethylene terephthalate (PET) tube (22 cm diameter, 50 cm

154 length, 25 μm thickness; Kalle UK Ltd, Witham, UK) at both ends. A valve was attached to the bags

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155 with Parafilm® (Bemis Company, Inc, Oshkosh, Wisconsin, USA) which allowed the exhaust bags to

156 be filled and stored prior to each experimental run and then be attached to the gas sampling pump of

157 the volatile delivery system of the artificial flower setup.

158

159 Experimental design

160 The two identical artificial flower setups were randomly assigned each day, on four different days, to

161 either location and to one of two treatments: fresh air or diesel exhaust. All four flower colors(black,

162 blue, red, and yellow) were used each day and the order they were presented was randomized. All

163 flowers were of the same color between treatments within one experimental run.

164 Before each experimental run, 1 µL of the above mentioned synthetic floral volatile blend was added

165 to a filter paper and placed into the gas washing bottle of the artificial flower setup. To each flower a

166 PCR tube filled with a 30% sucrose solution was added as a reward. One experimental run lasted 30

167 min (Fig. 2). For the first 15 min, volatiles were delivered at a flow rate of 0.5 L/min with fresh air for

168 both treatments. For the second 15 min, the flow rate remained the same but the volatile delivery in the

169 diesel exhaust treatment was switched to diesel exhaust. The diesel exhaust bags were swapped after

170 7.5 min. Throughout the experimental period of 30 min the sucrose solution was refilled every 10 min.

171 All four colors were run on one day and the experiment was repeated on four days and filmed with a

172 Veho Muvi HD and Hitachi Full HD DZHV 593E camcorder mounted on tripods.

173

174 Data analysis

175 For analysis, videos were played back using the VLC media player and data for one flower at a time

176 was recorded. A flower visit was noted when a worker honey bee landed on the flower and extended

177 her proboscis into the PCR tube holding the sucrose solution. For each flower, the visitation rate per

178 minute was recorded. In most instances the visitation rate (bees per flower per minute) was calculated

179 by dividing the total number of bees which visited a flower during the experimental periods of 15 min

180 by fifteen. However, on a few occasions individual bees accidentally removed the PCR tube with the

181 sugar solution from a flower and hence the reward was not offered for the total experimental period. In

7

182   these instances, the total number of bees, which have visited the flowers, were divided by the total

183   time that the sugar reward was actually offered.

184   Bee activity at the artificial flower set up was statistically analyzed using the R software environment

185   (version 3.4.3.; R Development Core Team 2018). A linear mixed-effects model from the *nlme*

186   package (Pinheiro et al. 2015) was fitted. The response variable was the number of bee visits per

187   minute in the second experimental period. Treatment, color, and their interaction were modelled as

188   fixed effects while the visitation rate of the first experimental period was included as a covariate. The

189   date of the visitation was modelled as a random effect. Visual inspection of the residual plots showed

190   no deviations from homoscedasticity and normality. Pairwise comparisons with a Tukey p-value

191   adjustment were conducted using the *pairs* method from the *lsmeans* package (Lenth 2016).

192

193   **Results**

194   Red flowers were visited significantly more than flowers of any other color ($F_{(3,116)} = 33.759$,

195    $P < 0.001$; Fig. 3A). Bee visits per minute in the first experimental period predicted bee visits of the

196   second period ($F_{(1,116)} = 52.879$, $P < 0.001$). Whether the flower volatiles were delivered by diesel or

197   fresh air did not influence the visitation rate ($F_{(1,116)} = 0.129$, $P = 0.719$; Fig. 3B). However, a

198   significant interaction of color and treatment influenced how many bees visited per minute ($F_{(3,116)} =$

199   9.339, $P < 0.001$; Fig. 3C). Red and black flowers were visited more in the fresh air treatment ($8.28 \pm$

200   0.66 SE, 6.32 ± 0.39 SE) compared to the diesel treatment (7.77 ± 0.59 SE, 5.45 ± 0.49 SE). For

201   yellow flowers both treatments were visited at an almost equal rate (clean air: 5.49 ± 0.58 SE, diesel:

202   5.46 ± 0.76 SE), but blue flowers were more attractive when the volatiles were delivered with diesel

203   exhaust (6.19 ± 0.75 SE) compared to delivery with fresh air (5.14 ± 0.59 SE).

204

205   **Discussion**

206   In a previous laboratory study, we found that when we exposed the same common flower volatiles

207   used in this study to diesel exhaust, the blend altered significantly. The amount of myrcene decreased,

208   β-ocimene became undetectable, and β-caryophyllene was transformed into its geometric isomer

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209   isocaryophyllene. In a behavioral assay (Proboscis Extension Response) we demonstrated that these

210   alterations reduce the ability of honey bees to recognize the floral blend (Lusebrink et al. 2015).

211   However, these previous results were not supported by those of the current field assay, which

212   suggested that whether this common flower volatile blend was delivered using fresh air or diesel

213   exhaust had no influence on the overall visitation rate to the artificial flowers by free-flying honey

214   bees. Whilst there were interactions with flower color, there was no consistent effect of diesel

215   treatment. Honey bee behavior may have been influenced by experience gained beforehand; the bees

216   having previously been trained through positive association to visit the artificial flowers' locations.

217   Floral odors are commonly used in long-range attraction (Raguso 2008) and are likely to be more

218   important for new flower location discovery than for revisitation of an established foraging site. Our

219   result suggests the possibility that the ability to learn the location of successful foraging sites during

220   periods of low air pollution could be sufficient to negate the predicted disruption to foraging that may

221   occur during high air pollution events. We encourage further studies that specifically address this new

222   hypothesis.

223   Generally, bees preferred the red flowers, which is an unexpected result since the trichromatic color

224   vision of honey bees peaks at a wavelength of 544 nm (green) outside of the red spectrum of human

225   vision (Daumer 1956; Peitsch et al. 1992). Chittka & Waser (1997) reported that the L-receptor of

226   honey bees, which is their long wavelength type photoreceptor, has an extended tail towards longer

227   wavelengths reaching zero at 650 nm and hence bees can perceive the color red. However, bees

228   usually prefer short wavelength stimuli and only exhibit a weak preference for some longer

229   wavelengths (Menzel 1967). Visual discrimination by free-flying honey bees does not seem to be an

230   absolute phenomenon (Avarguès-Weber et al. 2010) but is at least partly dependent on experience

231   (Reser et al. 2012), therefore the preference for the red flowers in our field assay could be related to

232   experiences the honey bees gained before the start of our experiment.

233   Additionally, the bees' foraging behavior was influenced by treatment when blue flowers were on

234   display. Bees visited more often when the volatiles were delivered by diesel exhaust. This might be

235   due to the fact that toluene, which is part of diesel exhaust (see Lusebrink et al. 2015 supplementary

236   material) is chemically similar to benzaldehyde, a flower volatile which occurs in 64% of seed plant

237　families (Knudsen et al. 2006). Riffel et al. (2014) showed that in *Maduca sexta* moths toluene elicited

238　a strong antennal response and activated the same olfactory sensory neurons that respond to

239　benzaldehyde. It is possible that the honey bees in our study could have recently foraged on blue

240　flowers which emitted benzaldehyde as part of their floral odor, like some *Petunia* species (Stuurman

241　et al. 2004), and hence learned to associate the color blue with benzaldehyde.

242　The interaction between treatment and color could also be explained by the innate color preference of

243　bees, since we only observed a lower visitation rate in the diesel exhaust treatment, when the flower

244　color on display was not favored by bees (black and red), but not when the other colors were

245　presented. Naïve honey bees favor blue flowers (ranging from UV-blue at around 410 nm to bee blue

246　at 430 nm; Giurfa et al. 1995), which corresponds with our blue foam petals and might explain why,

247　even when the odor signal was altered, the blue flowers were visited more frequently. The color

248　preference for blue is followed by a preference for "bee green" (around 530 nm; Giurfa et al. 1995),

249　which corresponds with our yellow flowers that were visited equally in both treatments. Similarly,

250　Gumbert (2000) showed that free-flying bumblebees, which have similar color preferences to honey

251　bees, revert to their innate preferences under changing circumstances.

252　The results of this study demonstrate that, in order to understand the potential ecological impacts of air

253　pollution on insect foraging behavior and success, it is crucial to study such interactions under field

254　conditions.

255

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Figures legends:

348

349 Figure 1: Artificial flower setup: A) general design, custom-made Perspex® box housing the volatile
350 delivery system, B) artificial flower, made of a glassware adapter with a Perspex® disk in the middle
351 and flower petals made of foam sheets and C) while in use in the field, covered with camouflage
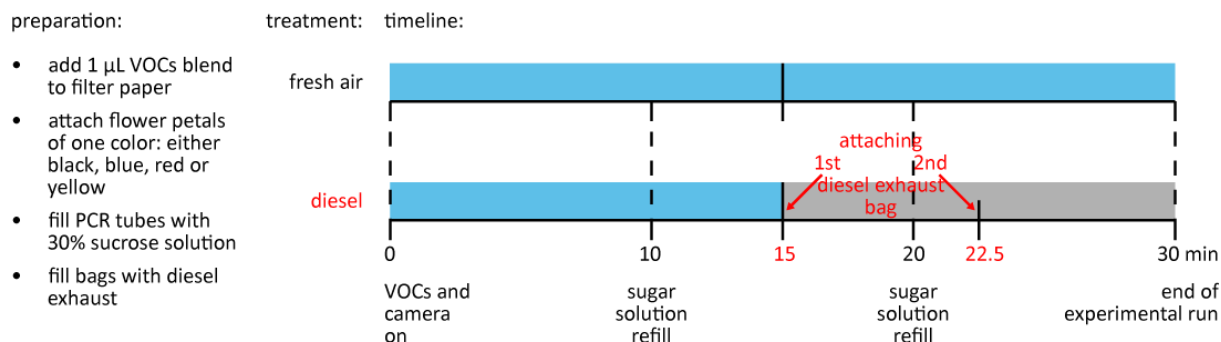352 netting and attached bag filled with diesel exhaust.

353

Figure 2: Preparation for and timeline of one experimental run: as preparation for each experimental run, 1 µL of VOCs blend was added to a filter paper and placed into the gas washing bottle of the VOCs delivery system. Flower petals of the same color were attached to the Perspex® disks of each flower (eight in total) and a PCR tube filled with a 30% sucrose solution was added to each flower. The diesel exhaust bags were filled with the exhaust from a diesel generator. At the start of each experimental run the camcorders and the gas sampling pump of the VOCs delivery system were turned on. For the first 15 min, VOCs were delivered with fresh air for both treatments. For the second 15 min the VOCs delivery in the diesel exhaust treatment was switched to diesel exhaust. During the experiment the sucrose solution was refilled every 10 min and the diesel exhaust bag was swapped after 7.5 min.
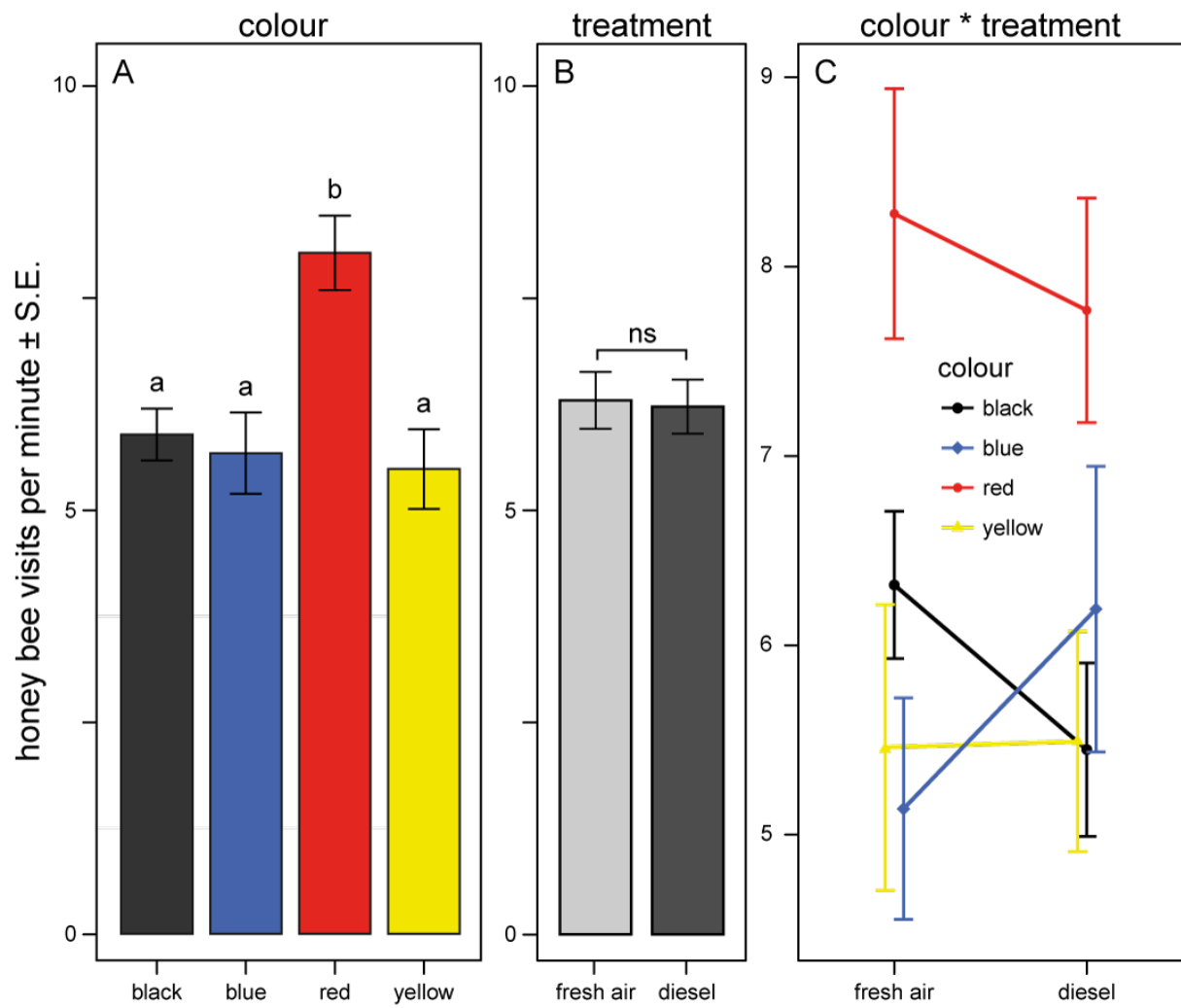
364

Figure 3: Bar graphs of main effects of A) color, different letters above the bars indicate statistically significant difference (P < 0.05) B) treatment, abbreviation ns: non-significant, and line graph of C) interactions of color and treatment on honey bee visits per minute at the artificial flower set up. The error bars represent standard errors (S.E.).

15