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## Diesel exhaust rapidly degrades floral odours used by honeybees

## SUBJECT AREAS:

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ECOSYSTEM SERVICES

ENVIRONMENTAL MONITORING

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**Honeybees utilise floral odours when foraging for flowers; we investigated whether diesel exhaust pollution could interrupt these floral odour stimuli. A synthetic blend of eight floral chemicals, identified from oilseed rape, was exposed to diesel exhaust pollution. Within one minute of exposure the abundances of four of the chemicals were significantly lowered, with two components rendered undetectable. Honeybees were trained to recognise the full synthetic odour mix; altering the blend, by removing the two chemicals rendered undetectable, significantly reduced the ability of the trained honeybees to recognize the altered odour. Furthermore, we found that at environmentally relevant levels the mono-nitrogen oxide (NO<sub>x</sub>) fraction of the exhaust gases was a key facilitator of this odour degradation. Such changes in recognition may impact upon a honeybee's foraging efficiency and therefore the pollination services that they provide.**

Chemical odours are central to communication in insects and their interaction with the environment<sup>1</sup>. A prime example of this is the floral odours that are produced by flowering plants to manipulate the behaviour of insects and facilitate pollination<sup>2,3</sup>. Globally the economic value of pollination has been estimated at €153 billion a year<sup>4</sup>, with 70% of the world's principal food crops relying upon pollination, equating to 35% of global food production<sup>5</sup>. Pollinator populations are declining on a global scale<sup>6</sup> and anthropogenic substances, such as synthetic insecticides, are implicated as key contributors to the reductions of both wild<sup>7,8</sup> and managed pollinators<sup>9–11</sup>.

Honeybees (*Apis mellifera*) are our most significant managed pollinator, yet every year significant numbers of honeybee colonies unexpectedly die worldwide<sup>6,9</sup>. The declines in managed honeybee populations have led to calls for further research to be conducted to enhance our understanding of honeybee health and well-being<sup>12</sup>. Current theory indicates that losses are most likely due to a combination/interaction of multiple factors<sup>9,10</sup>. However, whilst our comprehension of how these factors impact directly upon honeybee health is advancing, additional as yet undiscovered mechanisms are likely to be involved in honeybee declines.

Air pollution is one of the most ubiquitous environmental human impacts<sup>13</sup>, however its effects on honeybees are unknown. Honeybees have a sensitive sense of smell and an exceptional ability to learn and memorize new odours, enabling them to use floral odours to help locate, identify and recognise the flowers from which they forage<sup>14</sup>. There is a huge diversity of floral odours<sup>15</sup>, therefore any disruption to these blends could impact upon the ability of plants to communicate with their pollinators, which may have a negative impact on both parties. Theoretical models predict that anthropogenic emissions (including ozone, hydroxyl radicals and nitrate radicals) are likely to reduce the detection distances of plant emitted odours available to pollinators<sup>16</sup>, and empirical data has demonstrated that such compounds can interrupt plant-to-plant odour communication<sup>17</sup>.

Despite advances in filtration technology and tighter regulations on airborne emissions<sup>13</sup>, diesel exhaust remains a major environmental pollutant<sup>18</sup>. Many countries have guidelines in place to limit the emission of toxic gases produced as a result of the combustion of diesel and other fossil fuels (Table 1)<sup>13</sup>. Of these gases the NO<sub>x</sub> fraction is the most reactive and is known to have deleterious effects on both human health<sup>19</sup> and plant growth<sup>20</sup>. However the emissions limits for one of the NO<sub>x</sub> gases, nitrogen dioxide, are regularly exceeded especially in urban areas<sup>21</sup>. Whilst there is an overall downward trend in nitrogen dioxide emission in Europe<sup>21</sup>, it continues to be a significant environmental pollutant, particularly in countries undergoing rapid economic growth, such as China<sup>22</sup>.

We investigated whether diesel exhaust pollution alters the constituents of a synthetic floral odour blend, and if the highly reactive gases at concentrations down to environmentally relevant levels (100 ppb NO, 10 ppb NO<sub>2</sub>) were responsible for such changes and whether the changes elicited by this interaction could impair honeybee recognition of the floral blend.

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Table 1 | Ambient air quality standards for the major pollutant gases in diesel exhaust

	EU ambient air quality standards <sup>1</sup>		US ambient air quality standards <sup>2</sup>		Average maximum values used in diesel exposure experiments
	Averaging period	Concentration (abundance)	Averaging period	Concentration (abundance)	Concentration (abundance)
Nitrogen dioxide (NO <sub>2</sub> )	1 hour	200 µg/m <sup>3</sup> (100 ppb)	1 hour	200 µg/m <sup>3</sup> (100 ppb)	35.5 mg/m <sup>3</sup> (17.5 ppm)
	1 year	40 µg/m <sup>3</sup> (20 ppb)	1 year	107 µg/m <sup>3</sup> (53 ppb)	
Nitric oxide (NO)	Currently no standard		Currently no standard		26.2 mg/m <sup>3</sup> (19.8 ppm)
Carbon monoxide (CO)	8 hour	10 mg/m <sup>3</sup> (8 ppm)	1 hour	43 mg/m <sup>3</sup> (35 ppm)	280 mg/m <sup>3</sup> (227 ppm)
			8 hour	11 mg/m <sup>3</sup> (9 ppm)	
Sulphur dioxide (SO <sub>2</sub> )	1 hour	350 µg/m <sup>3</sup> (125 ppb)	1 hour	212 µg/m <sup>3</sup> (75 ppb)	None detected
	24 hour	125 µg/m <sup>3</sup> (44 ppb)	3 hour	1.4 mg/m <sup>3</sup> (500 ppb)	

<sup>1</sup>Source: EU directive 2008/50/EC - <http://ec.europa.eu/environment/air/quality/standards.htm> (accessed Jan 2012).

<sup>2</sup>Source: US EPA - <http://www.epa.gov/air/criteria.html> (accessed July 2012).

## Results

**Floral odour analysis – diesel exposures.** The natural floral odour from oilseed rape flowers (*Brassica napus*) (Fig. 1a) comprises a complex mix of chemicals. Our synthetic odour blend consisted of the 8 chemicals from this mix that elicit the strongest behavioural responses from honeybees<sup>23</sup>. The proportion of each chemical in our blend was designed to mimic the ratio at which they are naturally emitted from rape flowers (Fig. 1b and Supplementary Table 1). The blend was released into a sealed glass vessel that contained either ambient ‘clean’ air or air mixed with diesel exhaust. The volatile abundance of each chemical was then measured using gas chromatography-mass spectrometry (Supplementary Fig. 1). These diesel exhaust treatments contained high concentrations of NO<sub>x</sub> (nitric oxide and nitrogen dioxide) and carbon monoxide, however no sulphur dioxide was present (Table 1).

Within one minute  $\alpha$ -farnesene and  $\alpha$ -terpinene, which respectively constituted 72.5% and 0.8% of the original odour blend (Fig. 1b), were rendered undetectable in diesel polluted air and remained undetectable for the 2 hour study period (Fig. 1e, i & k and Supplementary Table 2). At every time point, two of the remaining six chemicals’ volatile abundances were significantly reduced by diesel exhaust compared with ambient air. Counter intuitively, the abundance of *p*-cymene was significantly increased by diesel exhaust after 60 minutes (Fig. 1f and Supplementary Table 3). All eight chemicals remained detectable after 2 hours in ambient air, indicating their relative stability in the absence of diesel exhaust and standard errors were tight across all time points demonstrating the consistency of the responses. Chemical abundances were often lowest at one minute, this was most pronounced amongst those chemicals that have lowest vapour pressures and therefore vaporize more slowly (Fig. 1g–j and Supplementary Table 1).

**Floral odour analysis – NO<sub>x</sub> exposures.** The NO<sub>x</sub> and diesel experiments were carried out using an identical design, except that instead of exhaust gases the glass vessel was filled with a combination of nitric oxide and nitrogen dioxide at two different ratios and at a range of different concentrations. The highest dose of NO<sub>x</sub> gases was to produce a worst case scenario, i.e. to replicate the concentrations and ratios measured in the diesel exhaust gas experiments. In order to investigate the relative impact of NO (which undergoes conversion to NO<sub>2</sub> under normal atmospheric conditions) the ratio of gases was skewed as far as possible to an NO enriched mixture. The range of doses were selected to range from those levels found in the diesel

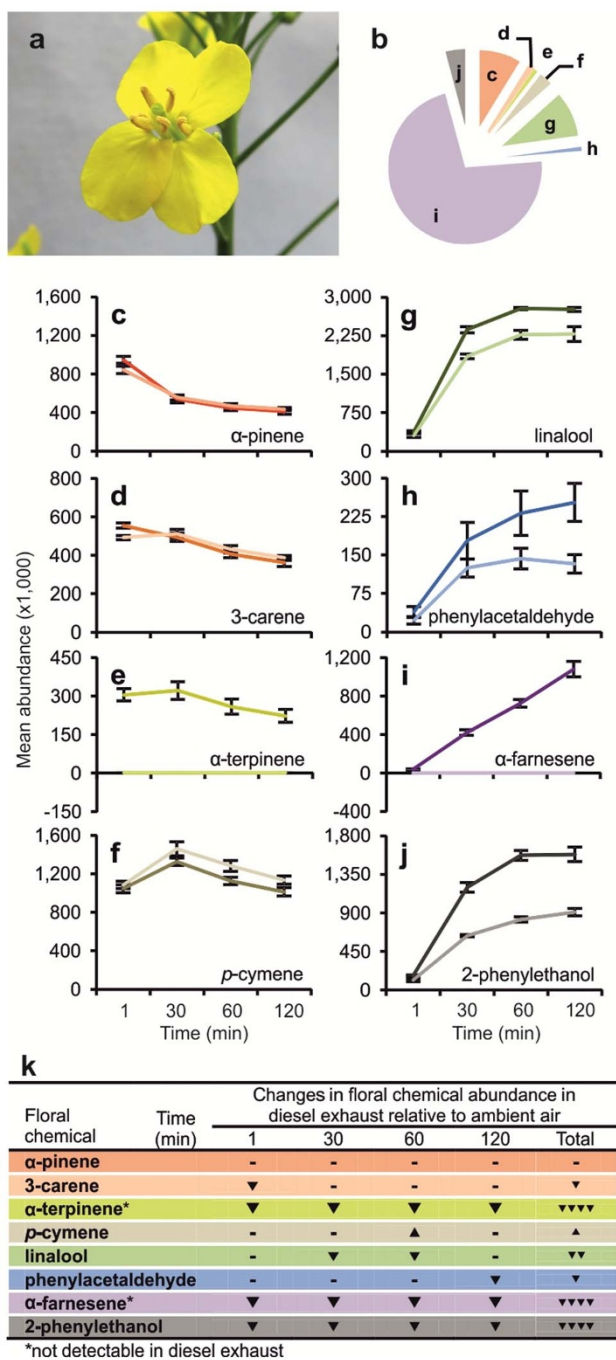
exhaust exposed sample, down to levels that are in line with the maximum recommended airborne levels under the current health guidance. In a 1 : 1 ratio at 10 ppm of each gas, approximately half the concentration used in the diesel exposure experiment,  $\alpha$ -terpinene was rendered undetectable,  $\alpha$ -farnesene was reduced by 97% and phenylacetaldehyde by 90% (Fig. 2, Supplementary Figs. 2–9 and Supplementary Table 5). Furthermore, in the 1 : 1 ratio at 0.1 ppm (i.e. 100 ppb), which is equivalent to the hourly average of nitrogen dioxide levels permitted by both EU and US air quality standards (Table 1), there were significant reductions in four of the eight components of the odour blend. Unexpectedly, exposure to NO<sub>x</sub> resulted in relative increases in the mean abundances of  $\alpha$ -pinene, 3-carene and *p*-cymene (Fig. 2 and Supplementary Figs. 2, 3 and 5).

**Proboscis extension reflex (PER).** We used the proboscis extension reflex of honeybees, where a honeybee extends its proboscis (Fig. 3b–c) when its antennae come into contact with sugar solution<sup>24</sup>, to train forager honeybees to associatively learn the synthetic floral odour blend. Trained honeybees should extend their proboscis when they next recognize the odour blend in the absence of reward. Honeybees were then presented with either the synthetic odour blend, or one of three artificially manipulated blends from which either  $\alpha$ -farnesene,  $\alpha$ -terpinene or both chemicals were omitted.

Removal of  $\alpha$ -farnesene, the major component in the synthetic odour blend, did not significantly reduce recognition relative to the full blend (Fig. 3d). In contrast, removal of  $\alpha$ -terpinene, a very minor component (0.8%) of the blend (Fig. 1b and Supplementary Table 1), significantly reduced recognition and when both chemicals were removed recognition dropped further.

## Discussion

Honeybee pollination can significantly increase the yield of crops, as typified by oilseed rape (Fig. 3a)<sup>26,27</sup> however, to forage effectively, honeybees need to be capable of learning and recognizing the plants floral odour blend<sup>2,28</sup>. Our results infer that a constituent of airborne pollutants, NO<sub>x</sub> gases, may be capable of disrupting the odour recognition process that odour guided pollinating insects rely on for location of floral food resources. Our experiments utilised higher total concentrations of NO<sub>x</sub> in the 1 : 1 than the 10 : 1 ratio experiments, because in producing the two different ratios the nitric oxide concentrations were kept constant, despite this the 1 : 1 ratio resulted in the greater reductions in abundances (Fig. 2 and Supplementary Tables 4 and 5), inferring that nitrogen dioxide may be more



**Figure 1** | The effects of diesel exhaust pollution upon the abundance of a synthetic oilseed rape floral odour blend. (a), An oilseed rape flower (photographed by RDG). (b), Percentages of each component of the synthetic floral blend, replicating the ratio at which they are naturally emitted from oilseed rape flowers. The colours and letters that represent each chemical are consistent throughout the figure. (c–j), Mean volatile abundances ( $\pm$ s.e.m) of the eight synthetic floral chemicals in ambient ‘clean’ air (darker lines) compared to their abundances in diesel exhaust polluted air (lighter lines) at four different times points after exposure ( $n = 5$ ). (k), The changes in mean floral chemical abundance in diesel exhaust polluted air relative to ambient air; which were either statistically significant ( $\blacktriangledown$ ;  $P \leq 0.05$ ), or in the case of both  $\alpha$ -terpinene and  $\alpha$ -farnesene where the chemical was no longer detectable in diesel exhaust polluted air ( $\blacktriangledown$ ). Directions of arrowheads indicate either increases or reductions in relative abundance.

involved than nitric oxide in the odour degradation process. As may be anticipated in a chemically reactive environment, one component of the blend, *p*-cymene, was detected at higher levels after  $\text{NO}_x$  exposure. This may be as a product of the known reaction between  $\alpha$ -terpinene and nitrates<sup>25</sup>.

Honeybees are known to use the whole range of chemicals found in a floral blend to discriminate between different blends<sup>29</sup>, our results indicate that some chemicals in a blend may be more important than others in this discrimination process. Whilst these results are the outcome of an artificial manipulation of the odour blend, the fact that removal of such a minor constituent can have such a profound effect on the ability of honeybees to recognize a floral odour may have significant ramifications for the ability of honeybees to efficiently forage for floral resources and therefore provide pollination services.

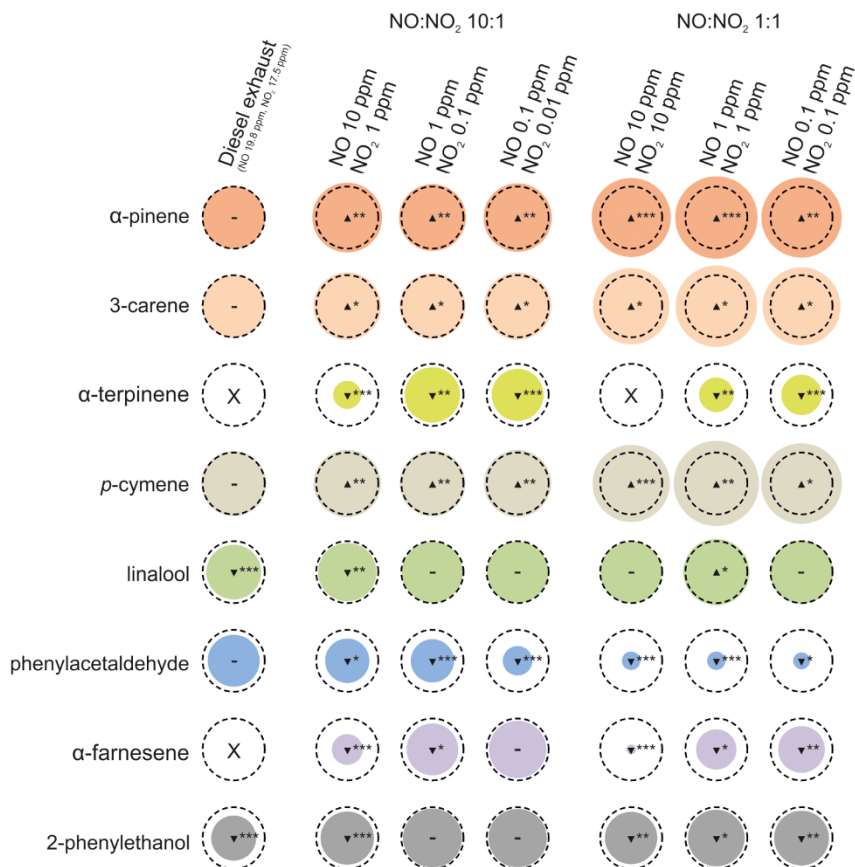
In nature honeybees use a combination of visual stimuli<sup>28</sup> and floral odours<sup>2</sup> to locate a flower for the first time. Honeybees associatively learn that a floral odour is concomitant with foraging success by gaining a reward of nectar whilst on the flower in the presence of high levels of floral odours<sup>24</sup>. Learning a floral odour remotely from the flower is less likely, because it would require a honeybee to remember an odour that occurs at a time distinct from the reward. Degradation of an odour source by pollution is likely to be more pronounced at distance from the flower, where concentrations of the odours are lower. Foraging honeybees may then be incapable of recognizing that the floral odours it detects remote from flowers are those that it associates with reward. This could result in a greater dependence upon other senses critical for foraging behaviour, such as vision, to compensate for the reduction in olfactory stimuli.

Disruption of odour communication by components of exhaust pollution could be detrimental to many insect species. In the case of pollinator species, including the honeybee, these effects would have major economic and ecological impacts, particularly when in conjunction with other stressors detrimental to pollinator health.

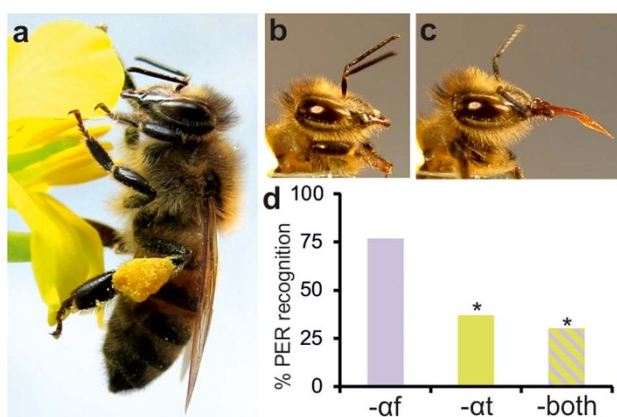
## Methods

**Floral odour collections.** Floral odour collections were made in cleaned 1 litre amber borosilicate glass bottles (VWR). For the diesel exhaust experiment, bottles were filled with either ambient air or diesel exhaust, collected at  $1 \text{ min}^{-1}$  for 3 min from a diesel generator’s exhaust (Suntom SDE 6500 E; Fuzhou). The diesel generator was run using a standard operating protocol of warm-up, engine load and time to first collection; the fuel and the engine oil were consistently purchased from the same supplier. The generator was maintained according to the manufacturers instructions. The concentrations of nitrogen dioxide ( $\text{NO}_2$ ), nitric oxide (NO), carbon monoxide (CO) and sulphur dioxide ( $\text{SO}_2$ ) produced at the generator’s exhaust were measured using a toxic gas probe (TG501+; Graywolf Sensing Solutions). For the  $\text{NO}_x$  experiments,  $\text{NO}_x$  for the 10 : 1  $\text{NO}:\text{NO}_2$  ratio were produced from a commercially purchased gas cylinder (BOC Group) and for the 1 : 1  $\text{NO}:\text{NO}_2$  ratio  $\text{NO}_x$  were produced by reducing nitric acid with elemental copper. Concentrations of 10 ppm, 1 ppm, 0.1 ppm per bottle were achieved by using gas tight syringes and volumetric calculations. Bottles were sealed with 2 layers of Parafilm® (Pechiney Plastic Packaging Company) and a GL45 cap (VWR). One microliter of the synthetic odour blend (Supplementary Table 1), applied to a 2.1 cm diameter filter paper (Grade 3 M), was placed into the glass bottle along with a stir bar (operated at 300 rpm to mix air). After 1, 30, 60 and 120 min (only after 30 min for  $\text{NO}_x$  experiments) of mixing a solid-phase microextraction fibre (SPME, blue fibre 65  $\mu\text{m}$  PDMS-DVB; Supelco) was inserted into the bottle through a 1 mm bore hole in the cap, for a 5 min exposure/adsorption period. For the diesel experiment, the process was repeated 5 times for both ambient air and diesel exhaust. For the  $\text{NO}_x$  experiments, the process was repeated 4 times for ambient air and 4 times for each  $\text{NO}:\text{NO}_2$  ratio and concentration.

**Floral odour analysis.** Chemicals were thermally desorbed from the SPME fibres in the injector ( $250^\circ\text{C}$ ) of a Hewlett-Packard 6890 gas chromatograph, coupled to a 5972 mass spectrometer. The carrier gas was helium ( $1 \text{ ml min}^{-1}$ ) and the injector was operated in a split mode (10 : 1). The capillary column was an HP-INNOWAX (30 m, 0.25 mm i.d., 0.25 mm film; Agilent Technologies). The oven temperature was held at  $50^\circ\text{C}$  for 2 min and then increased at  $5^\circ\text{C min}^{-1}$  to  $70^\circ\text{C}$  and then at  $10^\circ\text{C min}^{-1}$  to  $240^\circ\text{C}$ . The mass spectrometer ( $250^\circ\text{C}$ ) scanned from mass 350 to 40 at a rate of 2.43 times  $\text{s}^{-1}$  and data were captured and analysed by Enhanced Chemstation software (v. B.01.00; Agilent Technologies). The data for each chemical at each time point (or each  $\text{NO}_x$  concentration and ratio) were examined for the normality of their distributions using a series of Shapiro-Wilk tests and normal Q-Q plots. For those time points that



**Figure 2** | The effects of varying concentrations and ratios of NO and NO<sub>2</sub> upon the abundance of a synthetic oilseed rape floral odour blend. Circles indicate the percent change in mean abundances of the synthetic floral chemicals in ambient ‘clean’ air (dashed circle) compared to their abundances in either diesel exhaust polluted air, or air contaminated with NO and NO<sub>2</sub> (filled circles) at a ratio of 10:1 or 1:1, with NO at concentrations of 10, 1 or 0.1 ppm for both ratios ( $n = 4$ ). Abundances were measured after 30 minutes. Statistically significant changes in abundance are denoted by an arrowhead that indicates a significant increase or decrease (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ). An (X) indicates that the chemical was no longer detectable in those treatments.



**Figure 3** | Tests of honeybee recognition of synthetic odour blends. (a), A honeybee worker foraging on an oilseed rape flower (photographs by R Girling, C Reitmayer). (b–c), A honeybee worker (photographs by R Girling, C Reitmayer) restrained for a proboscis extension reflex (PER) assay with proboscis retracted (b) and extended (c). (d), The percentage of forager honeybees which, after learning the full synthetic floral blend, extended their proboscis (indicating recognition) when presented with the synthetic blend minus either  $\alpha$ -farnesene ( $-\alpha f$ ),  $\alpha$ -terpinene ( $-\alpha t$ ) or both chemicals ( $-\text{both}$ ). The data are expressed as the per cent PER recognition of each blend relative to the PER recognition of the full synthetic blend ( $n \geq 25$ ), where on average 93% of forager honeybees learnt the full blend. Asterisks indicate a significant reduction in PER recognition of that blend in comparison to the full synthetic blend ( $P < 0.05$ ).

were normally distributed, a series of unpaired two-tailed  $t$ -tests (SPSS v.19; IBM) were used to compare the mean abundances of each floral chemical between ambient air and diesel exhaust treatments. For each time point equal variances were assumed, unless Levene’s tests demonstrated that variances were not equal. For those time points that were not normally distributed two-tailed Mann-Whitney U tests were performed (Supplementary Table 3).

**Proboscis extension reflex (PER).** Honeybees, *Apis mellifera*, were from colonies kept at the University apiary (50° 56' 10"N, 1° 23' 39"W). For each assay, 30 returning forager honeybees (identified by full pollen baskets) were collected in individual plastic tubes between 14.00–16.00 BST. Honeybees were immobilized at 4°C, harnessed in 1 ml pipette tips<sup>30</sup>, fed to satiation with 30% sucrose solution and kept at 20°C. The morning after collection, honeybees were randomly assigned into groups of 7–10 individuals. Each honeybee was trained to associatively learn the synthetic odour blend. A harnessed honeybee was placed in a well-ventilated chamber in front of a flow of ambient air. After 10 s the honeybee was exposed to odours from a glass tube containing a 2.1 cm diameter filter paper impregnated with 8  $\mu$ l of the synthetic blend, after a further 10 s the air flow was switched back to ambient. Five seconds into the odour stimulus the honeybees’ antennae were touched with 30% sucrose solution and honeybees were allowed to feed for 10 s. Each honeybee underwent 6 exposures with 10 min intervals between each exposure. Honeybees which extended their proboscis (Fig. 2c) in response to the odour stimuli on the 6th exposure were considered to have learnt the blend and were used in recognition trials. In the recognition trials the groups of honeybees were tested to one of four odours, either the synthetic blend or a blend where  $\alpha$ -farnesene,  $\alpha$ -terpinene or both chemicals were omitted. Recognition mirrored the conditioning trials, with the omission of sucrose. Extension of the proboscis within 10 s in response to the onset of the odour stimulus was classified as a positive recognition. Responses to each of the three manipulated blends are expressed as the per cent PER recognition of each blend relative to the per cent PER recognition of the full synthetic blend. A  $\chi^2$  test was used to compare the total numbers of honeybees recognizing each odour between all four odour groups, combined with a  $z$ -test to perform pairwise comparisons between odour groups (SPSS v.19; IBM).



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## Author contributions

R.D.G., T.A.N. and G.M.P. conceived the study. I.L., R.D.G., T.A.N. and G.M.P. designed the experiments. I.L. performed the experiments and collected data for GC-MS studies. E.F. and I.L. performed the experiments and collected data for the PER tests. R.D.G. performed the analysis. R.D.G. and T.A.N. supervised data collection and wrote the manuscript. R.D.G. and I.L. contributed equally to the study. All authors discussed the results and commented on the manuscript.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

**Competing financial interests:** The authors declare no competing financial interests.

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