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Diesel exhaust rapidly degrades floral odours used by honeybees

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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 (NOx) 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 environment1. A prime example of this is the floral odours that are produced by flowering plants to manipulate the behaviour of insects and facilitate pollination2,3. Globally the economic value of pollination has been estimated at €153 billion a year4, with 70% of the world’s principal food crops relying upon pollination, equating to 35% of global food production5. Pollinator populations are declining on a global scale6 and anthropogenic substances, such as synthetic insecticides, are implicated as key contributors to the reductions of both wild7,8 and managed pollinators9–11.

Honeybees (Apis mellifera) are our most significant managed pollinator, yet every year significant numbers of honeybee colonies unexpectedly die worldwide11. 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-being12. Current theory indicates that losses are most likely due to a combination/interaction of multiple factors9,10. 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 impacts13, 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 forage14. There is a huge diversity of floral odours15, 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 pollinators16, and empirical data has demonstrated that such compounds can interrupt plant-to-plant odour communication17.

Despite advances in filtration technology and tighter regulations on airborne emissions13, diesel exhaust remains a major environmental pollutant18. 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)19. Of these gases the NOx fraction is the most reactive and is known to have deleterious effects on both human health19 and plant growth20. However the emissions limits for one of the NOx gases, nitrogen dioxide, are regularly exceeded especially in urban areas21. Whilst there is an overall downward trend in nitrogen dioxide emission in Europe21, it continues to be a significant environmental pollutant, particularly in countries undergoing rapid economic growth, such as China22.

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 NO2) were responsible for such changes and whether the changes elicited by this interaction could impair honeybee recognition of the floral blend.
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 honeybees23. 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 blend was designed to mimic the ratio at which they are naturally released into the atmosphere.

In a 1 : 1 ratio at 10 ppm of each gas, approximately half the concentration used in the diesel exposure experiment, α-terpinene was rendered undetectable, and 3-carene 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 NOx resulted in relative increases in the mean abundances of α-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 solution24, 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 α-farnesene, α-terpinene or both chemicals were omitted.

Removal of α-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 α-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–c) when its antennae come into contact with sugar solution24, to train forager honeybees to associatively learn the plants floral odour blend.29 Our results infer that a constituent of airborne pollutants, NOx 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 NOx in the 1 : 1 than the 10 : 1 ratio experiments, because in producing the two different ratios the nitrogen 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 toxic than nitrogen monoxide.

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

<table>
<thead>
<tr>
<th>EU ambient air quality standards</th>
<th>US ambient air quality standards</th>
<th>Average maximum values used in diesel exposure experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Averaging period</td>
<td>Concentration (abundance)</td>
<td>Averaging period</td>
</tr>
<tr>
<td>Nitrogen dioxide [NO2]</td>
<td>1 hour</td>
<td>200 µg/m³</td>
</tr>
<tr>
<td></td>
<td>1 year</td>
<td>40 µg/m³</td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td>Current no standard</td>
<td>Current no standard</td>
</tr>
<tr>
<td>Carbon monoxide (CO)</td>
<td>8 hour</td>
<td>10 mg/m³</td>
</tr>
<tr>
<td></td>
<td>8 hour</td>
<td>11 mg/m³</td>
</tr>
<tr>
<td>Sulphur dioxide (SO2)</td>
<td>1 hour</td>
<td>350 µg/m³</td>
</tr>
<tr>
<td></td>
<td>24 hour</td>
<td>125 µg/m³</td>
</tr>
</tbody>
</table>

Honeybees are known to use the whole range of chemicals found in a floral blend to discriminate between different blends, 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 and floral odours 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. 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 clean 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 l min⁻¹ for 3 min from a diesel generator’s exhaust (Suntom SDE 6500 E; Fujzou). 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 manufacturer’s instructions. The concentrations of nitrogen dioxide (NO₂), nitric oxide (NO), carbon monoxide (CO) and sulphur dioxide (SO₂) produced at the generator’s exhaust were measured using a toxic gas probe (TG501; Graywolf Sensing Solutions). For the NO₂ experiments, NOₓ for the 10:1 NO:NO₂ ratio were produced by using a commercially purchased gas cylinder (BOC Group) and for the 1:1 NO:NO₂ ratio NO₂ was 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 microfilm 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 NOₓ experiments) of mixing a synthetic oilseed rape floral 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 NOₓ experiments) of mixing a solid-phase microextraction fibre (SPME, blue fibre 65 μm 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 NOₓ experiments, the process was repeated 4 times for ambient air and 4 times for each NOₓ:NO ratio and concentration.

**Floral odour analysis.** Chemicals were thermally desorbed from the SPME fibres in the injector (250°C) of a Hewlett-Packard 6890 gas chromatograph, coupled to a 5972 mass spectrometer. The carrier gas was helium (1 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 μm film; Agilent Technologies). The oven temperature was held at 50°C for 2 min and then increased at 5°C min⁻¹ to 70°C and then at 10°C min⁻¹ to 240°C. The mass spectrometer (250°C) scanned from mass 350 to 40 at a rate of 2.43 times 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 NOₓ 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 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 NOₓ exposure. This may be as a product of the known reaction between α-terpinene and nitrates.

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 (± s.e.m) of the eight synthetic floral chemicals in ambient air. After 1, 30, 60 and 120 min (only after 30 min for NOₓ experiments) of mixing a synthetic oilseed rape floral 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 NOₓ experiments) of mixing a solid-phase microextraction fibre (SPME, blue fibre 65 μm 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 NOₓ experiments, the process was repeated 4 times for ambient air and 4 times for each NOₓ:NO ratio and concentration.

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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 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 tips30, 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 ml 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 α-farnesene, α-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 in comparison to the full synthetic blend (P < 0.05).

Figure 2 | The effects of varying concentrations and ratios of NO and NO$_2$ 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$_2$ (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 α-farnesene (-af), α-terpinene (-at) or both chemicals (-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 ≥ 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).

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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 the PER tests. R.D.G. performed the experiments. I.L. performed the experiments and collected data for GC-MS studies. E.F. 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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